

**UNITED STATES DISTRICT COURT
DISTRICT OF MASSACHUSETTS**

ASTELLAS INSTITUTE FOR REGENERATIVE)
MEDICINE, and STEM CELL & REGENERATIVE)
MEDICINE INTERNATIONAL, INC.,)

Plaintiffs,

V.

IMSTEM BIOTECHNOLOGY, INC., XIAOFANG WANG, and REN-HE XU,

Defendants.

C.A. NO. 1:17-cv-12239-ADB

**LEAVE TO FILE REPLY AND TO
FILE UNDER SEAL GRANTED ON
8/2/19 (Dkt. 80)**

**REPLY TO PLAINTIFFS' OPPOSITION TO
DEFENDANTS' MOTION FOR LEAVE TO FILE AMENDED COUNTERCLAIMS**

Pursuant to L.R. 7.1(b)(3) and Dkt. 80, defendants ImStem Biotechnology, Inc., Ren-He Xu, and Xiaofang Wang (collectively, “Defendants”) hereby reply to Plaintiffs’ Opposition to Defendants’ Motion for Leave to File Amended Counterclaims (Dkt. 75).

I. The ‘321 and ‘956 Counterclaims

With respect to the ‘321 patent and the ‘956 patent (for which Defendants are seeking to add more factual grounds for their existing claim), the Plaintiffs do not contend they were the first to invent the technical features underlying the proposed amended counterclaims. Instead, they make a series of heated accusations about bad faith and dilatory motives. Oppn. at 6.

There was no bad faith, no “lying in wait.” Well after the Complaint was filed (March 2018), counsel for Defendants told Dr. Lu that, at that time, Dr. Wang did not think (*i.e.* realize) that he had a claim to inventorship of the ‘321 patent. Oppn. Ex. 3 at 67:19-68:3. As Dr. Wang testified in his most recent (June 29, 2019) deposition, he only noticed the dependent claims in the ‘321 patent bearing his and Dr. Xu’s inventive contributions (and Drs. Kimbrel and Lanza’s plagiarism in the specification) in June 2019:

Q. So if you look at -- have you reviewed the ‘321 patent before?

A. Yes.

Q. And when have you reviewed the ‘321 patent?

A. So only until recently, and I think is after my deposition [on June 14, 2019] I carefully review this one. Before the deposition I know this patent, but I didn’t spend time to carefully review this.

Ex. 1 (Wang. Dep. Tr.) at 396:7-15.¹ Dr. Wang was not lying in wait; he simply had not focused on a patent *not in suit*. The significance of the ‘321 patent and its dependent claims only became apparent to him during (and after) his first deposition.² With respect to Dr. Xu (who lives in

¹ All exhibits are attached to the Declaration of Timothy R. Shannon in Support of Defendants’ Reply to Plaintiffs’ Opposition to Motion to Amend.

² Plaintiffs confuse (i) knowing inventive ideas at the time they were conceived, (ii) recalling those ideas 7 years later, and (iii) correlating those inventive ideas with the dependent claims in the ‘321 patent. (Oppn. at 7-9). Just

Macau and has been hard to communicate with throughout this case), he too focused on the claims in the ‘956 and ‘321 patents only when he came to the U.S. for his deposition and met his lawyers face-to-face for the first time (July 3, 2019).³ While not ideal, none of this merits an accusation of bad faith.

Further, the process of developing the case required reviewing and understanding Plaintiffs’ documents to determine when they allegedly conceived of various inventive ideas, *i.e.* before/after or with/without Drs. Wang and Xu. That process has been slowed by Plaintiffs having marked almost every one of their documents, “Highly Confidential,” under the Protective Order – an attorneys-eyes-only designation – making it impossible for Drs. Wang and Xu to help their lawyers review technical documents and spot issues. *See, e.g.*, Ex. 6 (designating Defendants’ own patent application as Highly Confidential).

The Court is not confronted with bad faith or dilatory motive but with the side effects of a size disparity. Plaintiffs – with in-house counsel, expert resources, a bottomless litigation budget, and more lawyers working on the case than ImStem has employees – are asking the Court to set an unreasonable standard.

The Plaintiffs’ “prejudice” argument falls flat. They have not identified anything they would have done differently during discovery. As they admit (Oppn. at 5), “the subject matter described in the ‘321 and ‘956 patents is the result of the same research project.” All of the relevant documents have been produced. Plaintiffs have taken all the depositions that they need. On July 29, 2019, they deposed Dr. Wang about, among other things, the proposed amended

because Drs. Wang and Xu knew, at the time, the inventive ideas they told Plaintiffs about does not mean they should have immediately understood their significance years later upon reviewing Plaintiffs’ other patents.

³ ImStem has eight employees, all scientists. None of its leadership is a native English speaker. None has experience with patent litigation. None has ever managed outside counsel.

counterclaims.⁴ Plaintiffs also knew about the new counterclaims (albeit shortly) prior to Dr. Xu's deposition (Oppn. at 15), and he repeatedly testified about the technical points. *See, e.g.*, Ex. 2 (Xu Dep. Tr.) at 178-179 (discussing both mitotic inactivation and IL-6). To the extent the Court finds that Dr. Xu should sit for a limited deposition, Defendants believe it can be accomplished via video from Macau without delay. The Court has not yet set a schedule for expert discovery, summary judgment, or pre-trial activities. Trial is nine months away: April 2020.⁵ A single clean-up deposition (if needed) would be far less burdensome than a new case.

The Plaintiffs' "futility" argument is in reality a motion to dismiss, on which they bear the burden of proof. Like their first motion, this one relies on non-HB-MS-C art to predict HB-MS-C inventions. *Cf.* Dkt. 37 at 9 ("[h]ere, none of the articles that Plaintiffs cite describes using hemangioblast-derived MSCs ..."). The new art and arguments again fail to demonstrate futility.

With respect to mitotic inactivation (the process of rendering the cells unable to reproduce themselves but still alive enough to secrete chemicals useful in treating *inter alia* multiple sclerosis), Plaintiffs have misstated the art and missed the framework shift that Drs. Wang and Xu contributed.⁶ *See* Oppn. at 13. For example, Çelebi *et al.* (Oppn. Ex. 11) concerns using MSCs as feeder cells, *in vitro*, to expand other cells. It has nothing to do with using the MSCs *in vivo* for clinical use, nor embryonic stem cell safety, as in the '321 and '956 patent claims. Indeed, Çelebi teaches that mitotic inactivation creates a "large divergence" in

⁴ Plaintiffs said they were reserving their arguments on the Motion, but asked many questions about the proposed amendments and ended the deposition with time to spare.

⁵ Defendants proposed a schedule to Plaintiffs immediately after the July 23, 2019 status conference. Plaintiffs have not yet responded. Moreover, Plaintiffs only recently identified a corporate designee and proposed an August 8th deposition date and have still not produced all damages-related documents for it.

⁶ *See* Oppn. at 13, citing Betül Çelebi et al., *Irradiated mesenchymal stem cells improve the ex vivo expansion of hematopoietic progenitors by partly mimicking the bone marrow endosteal environment*, 370 J. IMMUNOLOGICAL METHODS 93 (2011) (AIRM00297371) and Bocelli-Tyndall, Chiara, et al. *Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous-and allogeneic-stimulated lymphocytes in vitro*, *Rheumatology* 46.3 (2007): 403-408.

MSC secretion, *i.e.* arguably teaching *away* from Dr. Wang and Xu’s invention. *Id.* at AIRM00297376, 77 Fig. 4 (table showing “significant differences”). Likewise, Bocelli-Tyndall *et al.* (Oppn. Ex. 12) concerns bone-marrow MSCs and lymphocyte proliferation, not HB-MSCs, nor multiple sclerosis, nor tumorigenesis, nor *in vivo* use. *Id.* at 403.

Plaintiffs have likewise misunderstood Drs. Wang and Xu’s inventive contribution of IL-6. The ‘321 and ‘956 patents claims are not merely directed to measuring IL-6 (Oppn. at 12), but to using IL-6 as an indicator of sufficient-quality hES-MSCs vis-à-vis immunosuppressive function. None of the art cited by Plaintiffs (*id.*) links IL-6 levels with *in vivo* immunosuppressive efficacy. None concerns *in vivo* anti-inflammatory efficacy.

Plaintiffs’ argument that Drs. Wang and Xu should not be inventors because they merely “gather[ed] data points” and measure[ed] “innate characteristics” of HB-MSCs is also at odds with Plaintiffs’ own patenting practice. [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED] Plaintiffs’ dismissal of the Defendants’

inventive contributions is ironic, to say the least.

Finally, Plaintiffs’ novel argument against judicial economy – that a claim that was not litigated in one action (because it was raised “too late”) may be barred in another action on the ground that it was litigated in the first action (from which it was barred) – is not addressed by the cited cases. Oppn. at 14-15. It is also illogical. If the Court knocks out the proposed counterclaims, the relevant facts (*e.g.* mitotic inactivation, IL-6) will not be heard here, in this

action, as they efficiently should be.

II. The Statute of Limitations (“SOL”) Defense

Plaintiffs do not (because they cannot) deny that there is a factual basis for the SOL defense. Plaintiffs do not (because they cannot) advance any real “futility” argument. And their “prejudice” argument is conclusory; Plaintiffs claim – in a bald statement – that Defendants’ assertion of the defense “prejudiced [them] to gather discovery ... and consider [its] impact.” (Oppn. at 19). Yet, Plaintiffs never say what they would have done differently. All of the information is in their possession. The documents – which they produced and have known about for years – are clear. There is no one for them to depose. Expert discovery and summary judgment have not yet happened, and the parties are about nine months from trial. Consequently, the cases that Plaintiffs cite (*id.*) are distinguishable.⁷

Plaintiffs are left making heated *ad hominem* statements about motive. The facts do not justify the attacks. At the pleading stage, Defendants had no factual basis for asserting SOL – *i.e.* challenging the Plaintiffs’ statement that they “became aware of Defendants’ intended wrongful use of Plaintiffs’ confidential technical information around July 2015 [when the U.S. application published].”⁸ (Dkt. 1 at ¶ 62); Fed.R.Civ. P. 11.⁹ When Defendants prepared their

⁷ In *In re Cumberland Farms*, 284 F.3d 216, 225-27 (1st Cir. 2002), the motion for leave was made on the eve of trial (unlike here), with evidence the defense should have known a year prior (unlike here), and the opposing party identified “complex” legal issues that the new defense would implicate (unlike here). See also *Knapp Shoes, Inc. v. Sylvania Shoe Mfg. Corp.*, 15 F.3d 1222, 1226 (1st Cir. 1994) (defense made the last day of trial); *Jakobsen v. Mass. Port Auth.*, 520 F.2d 810, 813-14 (1st Cir. 1975) (defense raised *after* trial). Further, *Perlmutter v. Shazer* explicitly recognized that seeking to add a SOL defense via leave of court – as Defendants are seeking to do here – is entirely appropriate. 102 F.R.D. 245, 247 (D. Mass. 1984).

⁸ This allegation (and others like it) formed the basis of Plaintiffs’ as-pleaded state law claims, which mostly have a three year statute of limitations (November 13, 2014). See *id.* at ¶ 61 (Conversion); *id.* at ¶ 68 (Unjust Enrichment), *id.* at ¶¶ 73, 75 (M.G.L. ch 93A); *id.* at ¶ 80 (Misappropriation of Trade Secrets); *id.* at ¶¶ 84-85 (Negligent Misrepresentation).

⁹ Under Plaintiffs’ reasoning, a statute of limitations defense could *never* be added if the factual underpinnings are known only to (and obscured by) the plaintiff. This would turn the liberal standard for amending pleadings on its head. Fed. R. Civ. P. 15(a) (defense can be added “by leave of court”).

Answers (Dkt. 20, 33), discovery had not yet begun and all the facts were still in Plaintiffs' possession. Plaintiffs did not start producing documents until January 30, 2019, having delayed the case with a motion to dismiss. Over the next four months, they produced 313,000 pages of documents, from 15 different custodians, including 22,000 emails. All that material took time to review. Nor could the problem of review be solved by search terms alone; over 10,000 documents contained the term, "mesenchymal," for example. And buried in the total were 3,200 documents for which Plaintiff had failed to include complete metadata – an omission Defendants did not immediately spot. Defendants requested the missing metadata on June 19, 2019 and Plaintiffs turned it over the next day. *See* Ex. 5 (Email exchange).

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED] Put simply, Plaintiffs did not discover the Wang/Xu application in July 2015 as alleged (Dkt. 1 ¶ 62); [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

Indeed, discovery is *still* coming in that confirms all of this. On, July 29, 2019, after the

instant Motion was filed, Plaintiffs produced their privilege log. [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

In retrospect, counsel for Defendants could have, and perhaps should have, identified the SOL defense in the body of their opening brief rather than just the attached proposed counterclaims (bolded and blacklined). The request was nonetheless preserved, even if the initial argument and basis was first stated the next day in Court.¹⁰ But that oversight is not a “get out of jail free card” for Plaintiffs, who are trying to prevent the Court from even considering the SOL defense.¹¹ The only jury-triable claims are time-barred; this Court should hear the argument.

Dated: August 5, 2019

Respectfully submitted,

IMSTEM BIOTECHNOLOGY, INC., REN-
HE XU and XIAOFANG WANG,

By their Attorneys,

/s/ Timothy R. Shannon

Timothy R. Shannon, MA Bar # 655325

¹⁰ Status conference (Dkt. 73). Defendants apologized and offered to file a corrected brief to more fully explain the defense and flag individual documents; the Court said words to the effect that everyone was “now on notice.”

¹¹ Plaintiffs’ caselaw is inapposite. Not only is the procedural history in *Nikitine v. Wilmington Tr. Co.*, 715 F.3d 388, 390 (1st Cir. 2013) completely different, but Defendants here are seeking to add the SOL defense precisely because of “new allegations coming to light following discovery, or of previously unearthed evidence surfacing” (distinction the First Circuit has repeatedly used). *Id.* Likewise, *Redondo-Borges v. United States Dep’t. of Housing & Urban Dev.*, 421 F.3d 1, 6 (1st Cir. 2005) relates to a litigant’s failure to raise an issue in the trial court that it was pressing on appeal. Plaintiffs make the blanket statement that “new” arguments in a reply are waived. (Oppn. at 18.) This elevates form over substance. Defendants stated the basis in open court the next day. *Cf. United States v. Bradstreet*, 207 F.3d 76, 80 n. 1 (1st Cir. 2000) (“While a reply brief is not the proper place to raise new arguments, it is proper for a court to look there for clarification.”) (internal citations omitted); *see also Santos v. FEMA*, 327 F.Supp.3d 328, n. 10 (D. Mass. 2018) (despite party’s conclusory statement in its opening brief, court decided to consider the “fleshed out” arguments in supplemental briefing because of “the importance of the issues raised”).

VERRILL DANA LLP
One Portland Square
P.O. Box 586
Portland, Maine 04112-0586
(207) 774-4000
tshannon@verrilldana.com

Benjamin M. Stern, MA Bar # 646778
VERRILL DANA LLP
One Federal Street
Boston, Massachusetts 02108
(617) 309-2600
bstern@verrilldana.com

CERTIFICATE OF SERVICE

I hereby certify that I caused an unredacted copy of this document to be filed by hand as of the date below. A redacted copy will be filed in accordance with the Court's procedures through the CM/ECF system, to be sent electronically to the registered participants, and paper copies to be sent to those indicated as nonregistered participants.

Dated: August 5, 2019

/s/ Timothy R. Shannon
Timothy R. Shannon

**UNITED STATES DISTRICT COURT
DISTRICT OF MASSACHUSETTS**

ASTELLAS INSTITUTE FOR REGENERATIVE
MEDICINE, and STEM CELL & REGENERATIVE
MEDICINE INTERNATIONAL, INC.,

Plaintiffs,

V.

IMSTEM BIOTECHNOLOGY, INC., XIAOFANG
WANG, and REN-HE XU,

Defendants.

C.A. NO. 1:17-cv-12239-ADB

**LEAVE TO FILE UNDER SEAL
GRANTED ON 8/2/19 (Dkt. 80)**

**DECLARATION OF TIMOTHY R. SHANNON IN SUPPORT OF
DEFENDANTS' REPLY TO PLAINTIFFS' OPPOSITION TO DEFENDANTS'
MOTION FOR LEAVE TO FILE AMENDED COUNTERCLAIMS**

I, Timothy R. Shannon, state as follows:

1. I am an attorney at the firm Verrill Dana LLP, One Portland Square, Portland, ME 04101, counsel of record for the defendants, ImStem Biotechnology, Inc., Ren-He Xu, and Xiaofang Wang (collectively, the “Defendants”). I am over the age of 18, am fully competent to make this declaration, and make this declaration on personal knowledge.

2. Attached hereto as **Exhibit 1** are true and accurate copies of excerpts from the July 29, 2019 deposition of Xiaofang Wang, M.D., Ph.D.

3. Attached hereto as **Exhibit 2** are true and accurate copies of excerpts from the July 3, 2019 deposition of Ren-Xe Hu.

4. Attached hereto as **Exhibit 3** are true and accurate copies of excerpts from the June 18, 2019 deposition of Erin Kimbrel.

5. Attached hereto as **Exhibit 4** are true and accurate copies of excerpts from the June 24, 2019 deposition of Nicholas Arthur Kouris.

6. Attached hereto as **Exhibit 5** is a true and accurate copy of an email thread between myself and counsel for Plaintiffs, Lauren Sharkey, dated June 19-20, 2019.

7. Attached hereto as **Exhibit 6** is a true and accurate copy of an email and attachment Plaintiffs produced bearing Bates number AIRM00037250-387. Plaintiffs redacted and withheld the email (AIRM00037250) in their production.

8. Attached hereto as **Exhibit 7** is a true and accurate copy of an email and attachment Plaintiffs produced bearing Bates number AIRM00226256-AIRM00226393. Plaintiffs redacted and withheld the email (AIRM00226256) in their production. The attachment to the email (AIRM00226257-393) is identical to the document attached to the email in Ex. 6 (AIRM00037251-387). In an effort to streamline Defendants' submission, I only attached the first and last pages of AIRM00226257-393. If, however, the Court wishes to see Ex. 7 in its entirety, I would be happy to provide it.

9. Attached hereto as **Exhibit 8** is a true and accurate copy of a screenshot of metadata Plaintiffs produced (on or about June 20, 2019) for the emails and attachments at Exs. 6 & 7. This metadata was then loaded into my firm's document review software, and a screenshot was created therefrom.

10. Attached hereto as **Exhibit 9** is a true and accurate copy of an email Plaintiffs produced bearing Bates number AIRM00296171-72.

11. Attached hereto as **Exhibit 10** is a true and accurate copy of excerpted pages from Plaintiffs' privilege log, which was provided to Defendants on July 29, 2019.

12. Attached hereto as **Exhibit 11** is a true and accurate copy of U.S. Patent Application No. US 2015/2023820.

Signed under the pains and penalties of perjury this 5th day of August, 2019.

/s/ Timothy R. Shannon

Timothy R. Shannon

CERTIFICATE OF SERVICE

I hereby certify that I caused an unredacted copy of this document to be filed by hand as of the date below. A redacted copy will be filed in accordance with the Court's procedures through the CM/ECF system, to be sent electronically to the registered participants, and paper copies to be sent to those indicated as nonregistered participants.

Dated: August 5, 2019

/s/ Timothy R. Shannon

Timothy R. Shannon

EXHIBIT 1

HIGHLY CONFIDENTIAL

Wang, M.D., Ph.D., Xiaofang - Vol. II

July 29, 2019

264

UNITED STATES DISTRICT COURT

DISTRICT OF MASSACHUSETTS

NO. 1:17-cv-12239

ASTELLAS INSTITUTE FOR REGENERATIVE)	
MEDICINE, AND STEM CELL & REGENERATIVE)	
MEDICINE INTERNATIONAL, INC.,)	
Plaintiffs,)	
vs.)	
IMSTEM BIOTECHNOLOGY, INC., XIAOFANG)	
WANG, AND REN-HE XU,)	
Defendants.)	HIGHLY
)	CONFIDENTIAL

CONTINUED VIDEOTAPED DEPOSITION OF
XIAOFANG WANG, MD, PhD, called as a witness by and
on behalf of the Plaintiffs, pursuant to the
applicable provisions of the Federal Rules of Civil
Procedure, before P. Jodi Ohnemus, RPR, RMR, CRR,
CA-CSR #13192, NH-LSR #91, MA-CSR #123193, and
Notary Public, within and for the Commonwealth of
Massachusetts, at Latham & Watkins LLP, 200
Clarendon Street, Boston, Massachusetts, on Monday,
July 29, 2019, commencing at 1:13 p.m.

Henderson Legal Services, Inc.

202-220-4158

www.hendersonlegalservices.com

HIGHLY CONFIDENTIAL

Wang, M.D., Ph.D., Xiaofang - Vol. II

July 29, 2019

369

1 **models.**

2 Q. Let me show you what's been previously
3 marked as Exhibit 71. It's a copy of US Patent
4 8,963,321.

5 **A. (Witness reviews document.)**
6 **(Exhibit 71, previously marked.)**

7 Q. So if you look at -- have you reviewed the
8 '321 patent before?

9 **A. Yes.**

10 Q. And when have you reviewed the '321
11 patent?

12 **A. So only until recently, and I think is**
13 **after my deposition I carefully review this one.**
14 **Before the deposition I know this patent, but I**
15 **didn't spend time to carefully review this.**

16 Q. All right. So if you look at column 9.

17 **A. Which page?**

18 Q. It's -- if you look at the column numbers
19 at the top of the page, it has a number 9 at the
20 top.

21 **A. Uh-huh. (Witness reviews document.) Ah,**
22 **here.**

23 Q. Yeah. And you see there's a discussion of
24 the figures, figure 11, 11B, 12, and then figure
25 13?

EXHIBIT 2

CONFIDENTIAL

Xu, Ren-He

July 3, 2019

1

UNITED STATES DISTRICT COURT
DISTRICT OF MASSACHUSETTS

ASTELLAS INSTITUTE FOR)	
REGENERATIVE MEDICINE, AND)	
STEM CELL & REGENERATIVE)	
MEDICINE INTERNATIONAL, INC.,)	
)	
Plaintiffs,)	
)	
vs.)	C.A. No.
)	1:17-cv-12239
IMSTEM BIOTECHNOLOGY, INC.,)	
XIAOFANG WANG, and REN-HE XU,)	
)	
)	
Defendants.)	
_____)	

CONFIDENTIAL - PROTECTIVE ORDER

VIDEOTAPED DEPOSITION OF REN-HE XU, taken on
behalf of the Plaintiffs, at 12670 High Bluff Drive,
San Diego, California, commencing at 9:19 a.m. and
ending at 5:10 p.m., Wednesday, July 3, 2019.

Reported by:
Audrey L. Ricks
CSR No. 12098, CCR, RPR, CLR

Henderson Legal Services, Inc.

202-220-4158

www.hendersonlegalservices.com

CONFIDENTIAL

Xu, Ren-He

July 3, 2019

46 (Pages 178 to 181)

<p style="text-align: right;">178</p> <p>1 collaborative data, the data from us, especially the 2 in vivo data, in international conference. 3 Secondly, they had a plot to block our 4 publication. So our collaboration was, in my mind 5 was all about the publication in good quality 6 journal, but they had a plot to prevent that. And 7 they took -- they wanted to take all the credit. So 8 I feel unfortunate to collaborate with them. 9 BY MR. FRAZIER: 10 Q But you were given cells and a protocol; 11 correct? 12 MR. SHANNON: Objection. 13 THE WITNESS: I said I feel unfortunate. 14 If I didn't get the cells from them, I would have 15 our own TMSC method as well. 16 BY MR. FRAZIER: 17 Q But you didn't describe the TMS -- the 18 TMSC method in this grant application; right? 19 A At that time we didn't include the -- that 20 method. That was in -- under filing, I think, maybe 21 at that time. I'm not very clear. 22 First -- secondly, it's because, as I 23 said, no matter which method you make, the grant 24 application is the same. You -- you are targeting a 25 highly devastating disease in the west, and using a</p>	<p style="text-align: right;">180</p> <p>1 inhibit or inactivate the mitosis of the cells. The 2 cells still have effect. 3 This is very good news to patients. So 4 it's dramatically reduced the bio safety. You don't 5 need to concern the cells will proliferate in your 6 body. Those cells are from an ES cell line, right? 7 You don't want that. So this is great news. 8 BY MR. FRAZIER: 9 Q Other people had already 10 mitotically-inactivated MSC cells before using them 11 in animal models; correct? 12 A I don't think so. 13 Q Have you studied the literature on that? 14 A We searched around and we didn't see 15 similar reports before. 16 That's why I feel Xiaofang is a really 17 brilliant scientist. I was very fortunate to have 18 him as my postdoc, so he really had good background 19 in immunology training and also he capture a lot 20 from my stem cell research and developed very fast. 21 He's a very good scientist. 22 MR. FRAZIER: All right. Let me show you 23 what's been previously marked as Exhibit 50. 24 (Previously marked Exhibit 50 25 reviewed by the witness.)</p>
<p style="text-align: right;">179</p> <p>1 new cell type. So that's -- and especially this new 2 cell type we found it has much lower 3 pro-inflammatory factor IO6 and we could also use 4 mitotically-inactivated cells for the treatment. 5 These are all blessing, you know, for patients. So 6 that's -- those ideas matter. 7 Also you use MSC to treat the disease, 8 there's already tons of papers, tons of ideas. But 9 to use this ES-derived MSC to treat this disease 10 with low prolif -- pro-inflammatorous cytokine IO6 11 and mitotically-inactivated cells instead of 12 mitotically-active cells, this significantly reduced 13 the bio safety concern. 14 Q Right. And -- and you obviously were not 15 the first one to come up with the idea of 16 mitotically inactivating stem cells; correct? 17 A Mitotically inactivating cells is not new, 18 but mitotically inactivated ES-derived MSC is new. 19 It's important. 20 Q It was only new because they were a new 21 cell type as taught to you by ACT; right? 22 MR. SHANNON: Objection. 23 THE WITNESS: It's new because we tell -- 24 clearly tell people that mitotically inactivation 25 doesn't matter for the cell effects. So you can</p>	<p style="text-align: right;">181</p> <p>1 BY MR. FRAZIER: 2 Q My question is, do you recognize what 3 Exhibit 50 is? 4 A No. First time to me. 5 Q You haven't seen this one before? 6 A No. 7 Q Okay. All right. Are you aware that 8 there were various presentations of information made 9 to potential investors in ImStem? 10 A I knew they were looking for some more 11 investment. Yeah. 12 Q All right. But you didn't review either 13 the -- the business plan or the slides that are 14 attached to Exhibit 50? 15 A No. 16 MR. FRAZIER: Let's see. There's another 17 one in the stack there in front of you that is 18 Exhibit 66. 19 (Previously marked Exhibit 66 20 reviewed by the witness.) 21 BY MR. FRAZIER: 22 Q You see Exhibit 66 is another collection 23 of e-mails, this time from August of 2013? 24 A Yeah. There's -- it's talking about mice. 25 Q Yeah. So you see it begins on page 31134</p>

EXHIBIT 3

FILED UNDER SEAL

**Leave to File Under Seal Granted on August 2, 2019
(Dkt. No. 80)**

EXHIBIT 4

FILED UNDER SEAL

**Leave to File Under Seal Granted on August 2, 2019
(Dkt. No. 80)**

EXHIBIT 5

Ben Stern

From: Lauren.Sharkey@lw.com
Sent: Thursday, June 20, 2019 1:37 AM
To: Timothy Shannon; Rebecca.Rabenstein@lw.com; David.Frazier@lw.com; ASTELLASASTELLASV.IMSTEM.LWTEAM@lw.com; spritchett@nixonpeabody.com; shansen@nixonpeabody.com; tlieberman@nixonpeabody.com
Cc: Jennifer Harrell; Ben Stern
Subject: RE: Astellas v. ImStem, No. 1:17-cv-12239-ADB (D. Mass.) - Missing metadata
Attachments: CustodianOL.zip

Tim,

Attached is an overlay that has custodian, bates and file name information for the redacted documents. The password for this zip file is &38@dkwW. We are working on obtaining the additional metadata and will get it to you via another overlay as quickly as possible. We note that the first time you raised this issue was on June 19 at 11:14 pm, despite the fact that we produced documents to you on :

Production Volume	Date Produced
0001	January 30, 2019
0002	February 8, 2019
0003	February 19, 2019
0004 & 0004a	March 29, 2019
0005 & 0006	April 23, 2019
0007	June 13, 2019

Kind regards,

Lauren

From: Sharkey, Lauren (CH) <Lauren.Sharkey@lw.com>
Sent: Wednesday, June 19, 2019 10:57 PM
To: Timothy Shannon <tshannon@verrilldana.com>; Rabenstein, Reba (DC) <Rebecca.Rabenstein@lw.com>; Frazier, David (DC) <David.Frazier@lw.com>; #C-M ASTELLAS - ASTELLAS V. IMSTEM - LW TEAM <ASTELLASASTELLASV.IMSTEM.LWTEAM@lw.com>; 'spritchett@nixonpeabody.com' <spritchett@nixonpeabody.com>; 'shansen@nixonpeabody.com' <shansen@nixonpeabody.com>; 'tlieberman@nixonpeabody.com' <tlieberman@nixonpeabody.com>
Cc: Jennifer Harrell <jharrell@verrilldana.com>; Ben Stern <bstern@verrilldana.com>
Subject: RE: Astellas v. ImStem, No. 1:17-cv-12239-ADB (D. Mass.) - Missing metadata

Tim,

This is the first I am hearing of this- -could you provide me the bates of an example or two so I can look into the issue?

Thank you,

Lauren

From: Timothy Shannon <tshannon@verrilldana.com>

Sent: Wednesday, June 19, 2019 10:14 PM

To: Sharkey, Lauren (CH) <Lauren.Sharkey@lw.com>; Rabenstein, Reba (DC) <Rebecca.Rabenstein@lw.com>; Frazier, David (DC) <David.Frazier@lw.com>; #C-M ASTELLAS - ASTELLAS V. IMSTEM - LW TEAM <ASTELLASASTELLASV.IMSTEM.LWTEAM@lw.com>; 'spritchett@nixonpeabody.com' <spritchett@nixonpeabody.com>; 'shansen@nixonpeabody.com' <shansen@nixonpeabody.com>; 'tlieberman@nixonpeabody.com' <tlieberman@nixonpeabody.com>

Cc: Jennifer Harrell <jharrell@verrilldana.com>; Ben Stern <bstern@verrilldana.com>

Subject: Astellas v. ImStem, No. 1:17-cv-12239-ADB (D. Mass.) - Missing metadata

Lauren & Reba,

It has come to our attention that the Plaintiffs removed metadata from approximately 3200 redacted documents in the Astellas production. This includes both relevancy redactions and privilege redactions. By way of example, the custodian field is blank for over 3,000 documents. We consider this improper and assume it was an oversight. For documents containing relevancy redactions, the document by definition contains at least some responsive information; the metadata is associated with that responsive information. For documents containing privilege redactions, the document likewise contains non-privileged and responsive information, for which the metadata may be necessary to confirm privilege.

This is causing several problems. Most immediately, it's causing us to miss documents in our filtering, searching, analysis, and preparation for depositions. Please provide overlay load files containing the metadata fields agreed upon in the Court's ESI order ASAP. If you provide it by tomorrow morning we will endeavor to run our searches for Lanza in time to use them in the deposition.

Regards,

Tim

Timothy R. Shannon, Partner

One Portland Square

Portland, ME 04112-0586

Office: (207) 253-4622

One Boston Place, Suite 1600

Boston, MA 02108-4407

Office: (617) 309-2600

Bio: verrilldana.com/tshannon

Verrill Dana

This email and any attachment was sent from the law firm Verrill Dana, LLP. It may contain information that is privileged and confidential. If you suspect that you were not intended to receive it, please delete it and notify us as soon as possible. Thank you.

EXHIBIT 6

FILED UNDER SEAL

**Leave to File Under Seal Granted on August 2, 2019
(Dkt. No. 80)**

EXHIBIT 7

FILED UNDER SEAL

**Leave to File Under Seal Granted on August 2, 2019
(Dkt. No. 80)**

EXHIBIT 8

PCT Application in Astellas' production:

Search Results Search Criteria

DOCUMENTS: All Documents | actions | coding forms: Review Coding (Natives) | edit

Field	Value
EVDXID	AIRM00037250
BegAttach (Include Family)	AIRM00037250
DocType	BegAttach (Include Family)
Atty Notes	
DateSort	02-21-2014 12:00 AM
Custodian	Kimbrel, Erin
Email From	Fleishell, Mary Lee[Non-Employee]
Email To	Kimbrel, Erin <erin.kimbrel@astellas.com>;
Email Subject	The other IMStem PCT application
DateSent	02-21-2014
TimeSent	14:50:14
FileName	
Hidden Properties	
Search Hits	
Prod BegBates	AIRM00037250
Prod EndBates	AIRM00037250
DateCreated	MM-dd-yyyy
DateLastMod	MM-dd-yyyy

1 of 1 items per page

RELATIONSHIPS

Family

EVDXID	DocType	DateSort	Email From	DateSent	FileName	Tag Group: Doc Review De
AIRM00037250		02-21-2014 12:00 AM	Fleishell, Mary Lee[Non-Employee]	02-21-2014		

Document Details | AIRM-001

Withheld as Privilege

AIRM00037250 | 1 of 1

Native Viewer | Image | Production | Extracted Text | Transcript

1 of 1

Search Results

Search Criteria

DOCUMENTS: All Documents

actions

RECORD

edit

coding forms: Review Coding (Natives)

Field

Value

EVDXID

AIRM00037251

BegAttach (Include Family)

AIRM00037250

DocType

Atty Notes

DateSort

02-21-2014 12:00 AM

Custodian

Kimbrel, Erin

Email From

Email To

Email Subject

DateSent

TimeSent

FileName

WO14011407 (HB-MSC).pdf

Hidden Properties

Search Hits

Prod BegBates

AIRM00037387

Prod EndBates

AIRM00037387

DateCreated

01-20-2014

DateLastMod

02-17-2014

items per page

25

2 of 2

RELATIONSHIPS

Return To Document Set

Family

EVDXID

DocType

DateSort

Email From

DateSent

FileName

Tag Group: Doc Review Designatic

AIRM00037250

02-21-2014 12:00 AM

Fleishell, Mary Lee[Non-Employee]

02-21-2014

AIRM00037251

02-21-2014 12:00 AM

WO14011407 (HB-MSC).pdf

Document Details

AIRM-001

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau

(43) International Publication Date 16 January 2014 (16.01.2014)

(48) International Publication Number WO 2014/011407 A2

(51) International Patent Classification: A61K 48/00 (2006.01) C12N 1/68 (2006.01)

(21) International Application Number: PCT/US2013/048251

(22) International Filing Date: 27 June 2013 (27.06.2013)

(23) Priority Date: 12 July 2012 (12.07.2012)

(24) Priority Date: 11 February 2013 (11.02.2013)

(25) Priority Date: 01 February 2013 (01.02.2013)

(26) Priority Date: 01 February 2013 (01.02.2013)

(27) Priority Date: 01 February 2013 (01.02.2013)

(28) Priority Date: 01 February 2013 (01.02.2013)

(29) Priority Date: 01 February 2013 (01.02.2013)

(30) Priority Date: 01 February 2013 (01.02.2013)

(31) Priority Date: 01 February 2013 (01.02.2013)

(32) Priority Date: 01 February 2013 (01.02.2013)

(33) Priority Date: 01 February 2013 (01.02.2013)

(34) Priority Date: 01 February 2013 (01.02.2013)

(35) Priority Date: 01 February 2013 (01.02.2013)

(36) Priority Date: 01 February 2013 (01.02.2013)

(37) Priority Date: 01 February 2013 (01.02.2013)

(38) Priority Date: 01 February 2013 (01.02.2013)

(39) Priority Date: 01 February 2013 (01.02.2013)

(40) Priority Date: 01 February 2013 (01.02.2013)

(41) Priority Date: 01 February 2013 (01.02.2013)

(42) Priority Date: 01 February 2013 (01.02.2013)

(43) Priority Date: 01 February 2013 (01.02.2013)

(44) Priority Date: 01 February 2013 (01.02.2013)

(45) Priority Date: 01 February 2013 (01.02.2013)

(46) Priority Date: 01 February 2013 (01.02.2013)

(47) Priority Date: 01 February 2013 (01.02.2013)

(48) Priority Date: 01 February 2013 (01.02.2013)

(49) Priority Date: 01 February 2013 (01.02.2013)

(50) Priority Date: 01 February 2013 (01.02.2013)

(51) Priority Date: 01 February 2013 (01.02.2013)

(52) Priority Date: 01 February 2013 (01.02.2013)

(53) Priority Date: 01 February 2013 (01.02.2013)

(54) Priority Date: 01 February 2013 (01.02.2013)

(55) Priority Date: 01 February 2013 (01.02.2013)

(56) Priority Date: 01 February 2013 (01.02.2013)

(57) Priority Date: 01 February 2013 (01.02.2013)

(58) Priority Date: 01 February 2013 (01.02.2013)

(59) Priority Date: 01 February 2013 (01.02.2013)

(60) Priority Date: 01 February 2013 (01.02.2013)

(61) Priority Date: 01 February 2013 (01.02.2013)

(62) Priority Date: 01 February 2013 (01.02.2013)

(63) Priority Date: 01 February 2013 (01.02.2013)

(64) Priority Date: 01 February 2013 (01.02.2013)

(65) Priority Date: 01 February 2013 (01.02.2013)

(66) Priority Date: 01 February 2013 (01.02.2013)

(67) Priority Date: 01 February 2013 (01.02.2013)

(68) Priority Date: 01 February 2013 (01.02.2013)

(69) Priority Date: 01 February 2013 (01.02.2013)

(70) Priority Date: 01 February 2013 (01.02.2013)

(71) Priority Date: 01 February 2013 (01.02.2013)

(72) Priority Date: 01 February 2013 (01.02.2013)

(73) Priority Date: 01 February 2013 (01.02.2013)

(74) Priority Date: 01 February 2013 (01.02.2013)

(75) Priority Date: 01 February 2013 (01.02.2013)

(76) Priority Date: 01 February 2013 (01.02.2013)

(77) Priority Date: 01 February 2013 (01.02.2013)

(78) Priority Date: 01 February 2013 (01.02.2013)

(79) Priority Date: 01 February 2013 (01.02.2013)

(80) Priority Date: 01 February 2013 (01.02.2013)

(81) Priority Date: 01 February 2013 (01.02.2013)

(82) Priority Date: 01 February 2013 (01.02.2013)

(83) Priority Date: 01 February 2013 (01.02.2013)

(84) Priority Date: 01 February 2013 (01.02.2013)

(85) Priority Date: 01 February 2013 (01.02.2013)

(86) Priority Date: 01 February 2013 (01.02.2013)

(87) Priority Date: 01 February 2013 (01.02.2013)

(88) Priority Date: 01 February 2013 (01.02.2013)

(89) Priority Date: 01 February 2013 (01.02.2013)

(90) Priority Date: 01 February 2013 (01.02.2013)

(91) Priority Date: 01 February 2013 (01.02.2013)

(92) Priority Date: 01 February 2013 (01.02.2013)

(93) Priority Date: 01 February 2013 (01.02.2013)

(94) Priority Date: 01 February 2013 (01.02.2013)

(95) Priority Date: 01 February 2013 (01.02.2013)

(96) Priority Date: 01 February 2013 (01.02.2013)

(97) Priority Date: 01 February 2013 (01.02.2013)

(98) Priority Date: 01 February 2013 (01.02.2013)

(99) Priority Date: 01 February 2013 (01.02.2013)

(100) Priority Date: 01 February 2013 (01.02.2013)

EXHIBIT 9

FILED UNDER SEAL

**Leave to File Under Seal Granted on August 2, 2019
(Dkt. No. 80)**

EXHIBIT 10

FILED UNDER SEAL

**Leave to File Under Seal Granted on August 2, 2019
(Dkt. No. 80)**

EXHIBIT 11



US 20150203820A1

(19) **United States**(12) **Patent Application Publication**
Wang et al.(10) **Pub. No.: US 2015/0203820 A1**(43) **Pub. Date: Jul. 23, 2015**(54) **MESENCHYMAL-LIKE STEM CELLS
DERIVED FROM HUMAN EMBRYONIC
STEM CELLS, METHODS AND USES
THEREOF**(71) Applicant: **IMSTEM BIOTECHNOLOGY, INC.**,
Unionville, CT (US)(72) Inventors: **Xiaofang Wang**, Unionville, CT (US);
Ren-He Xu, Farmington, CT (US)(21) Appl. No.: **14/413,290**(22) PCT Filed: **Jun. 27, 2013**(86) PCT No.: **PCT/US13/48291**

§ 371 (c)(1),

(2) Date: **Jan. 7, 2015****Publication Classification**(51) **Int. Cl.****C12N 5/0775** (2006.01)**C12N 15/01** (2006.01)**A61K 35/28** (2006.01)**C12N 13/00** (2006.01)(52) **U.S. Cl.**CPC **C12N 5/0662** (2013.01); **C12N 13/00**
(2013.01); **C12N 15/01** (2013.01); **A61K 35/28**
(2013.01); **C12N 2501/155** (2013.01); **C12N**
2501/165 (2013.01); **C12N 2501/10** (2013.01)

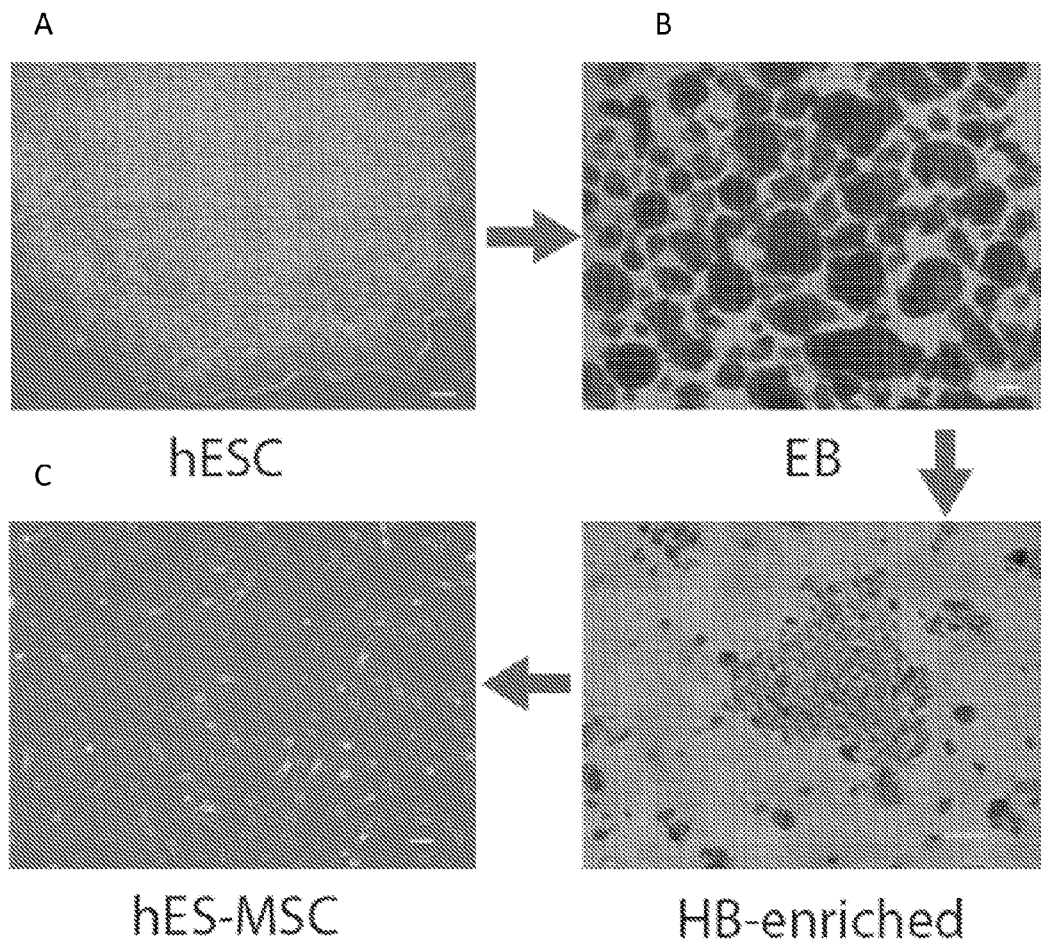
(57)

ABSTRACT

The present invention relates to methods of generating and expanding human embryonic stem cell derived mesenchymal-like stem/soma cells. These hES-MSCs are characterized at least in part by the low level of expression of IL-6. These cells are useful for the prevention and treatment of T cell related autoimmune disease, especially multiple sclerosis, as well as for delivering agents across the blood-brain barrier and the blood-spinal cord barrier. Also provided is a method of selecting clinical grade hES-MSC and a method of modifying MSC to produce a MSC with specific biomarker profile. The modified MSC are useful for treatment of various diseases.

Related U.S. Application Data

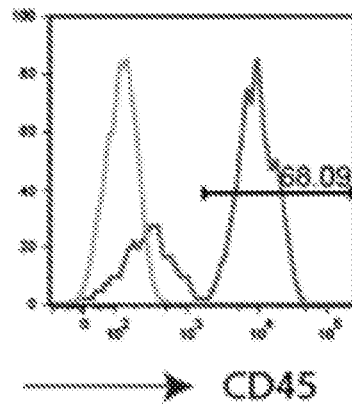
(60) Provisional application No. 61/762,961, filed on Feb. 11, 2013, provisional application No. 61/670,787, filed on Jul. 12, 2012.



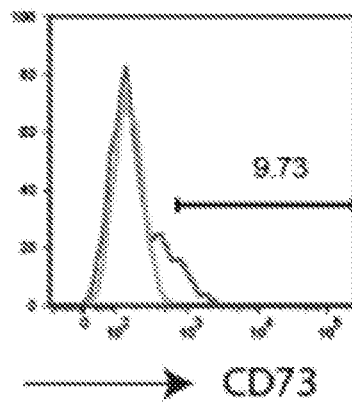
FIGS. 1 (A-D)

Day 9 Hemangioblast

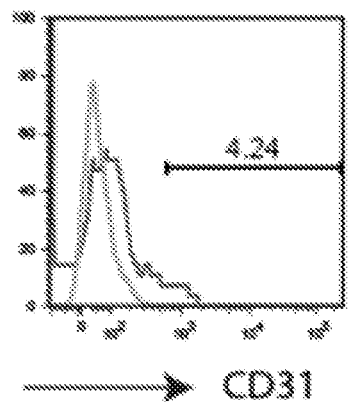
A



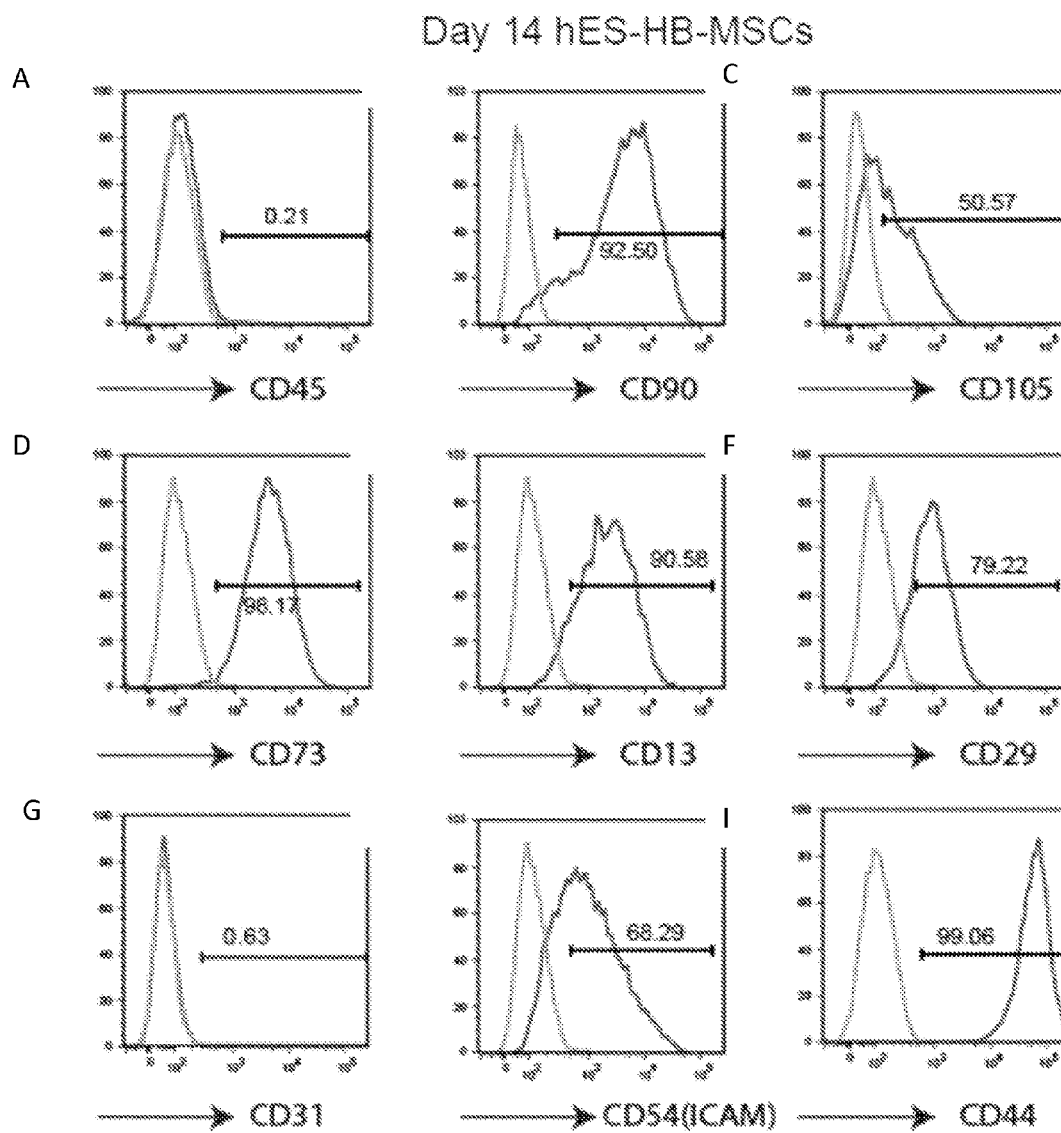
B



C



FIGS. 2 (A-C)



FIGS. 3 (A-I)

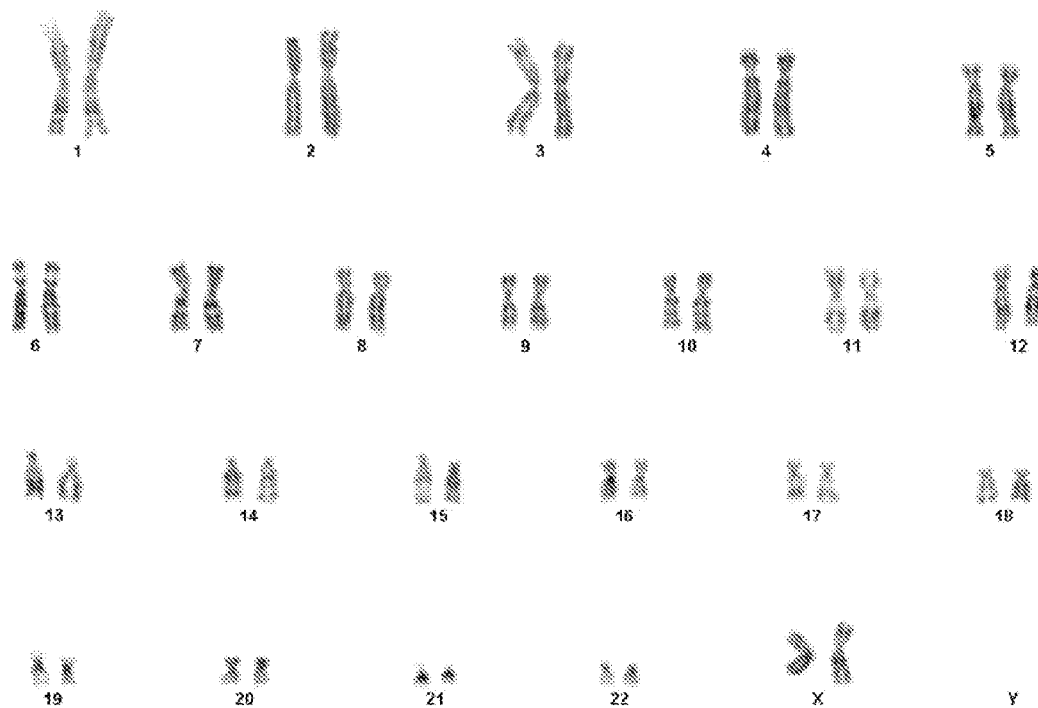
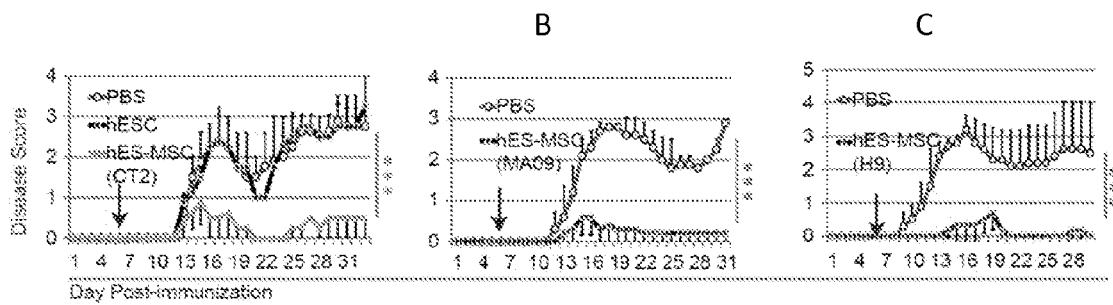
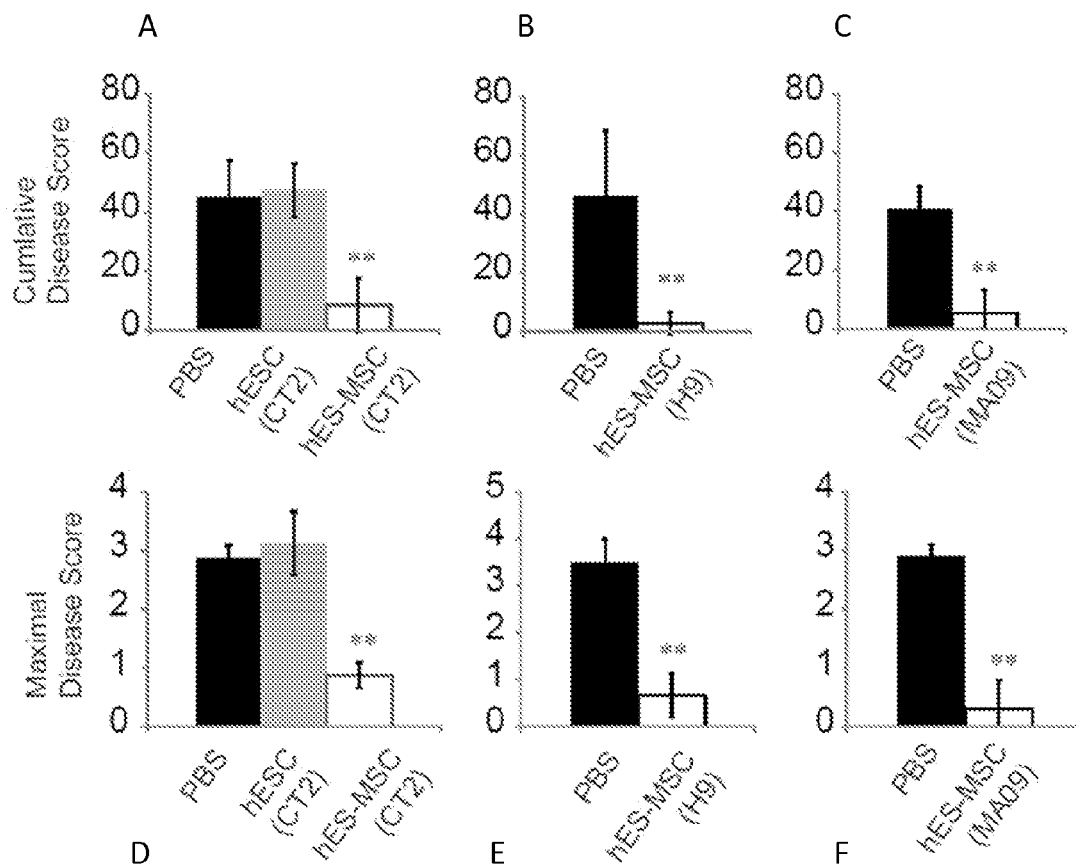


FIG. 4



FIGS. 5 (A-C)



FIGS. 6 (A-F)

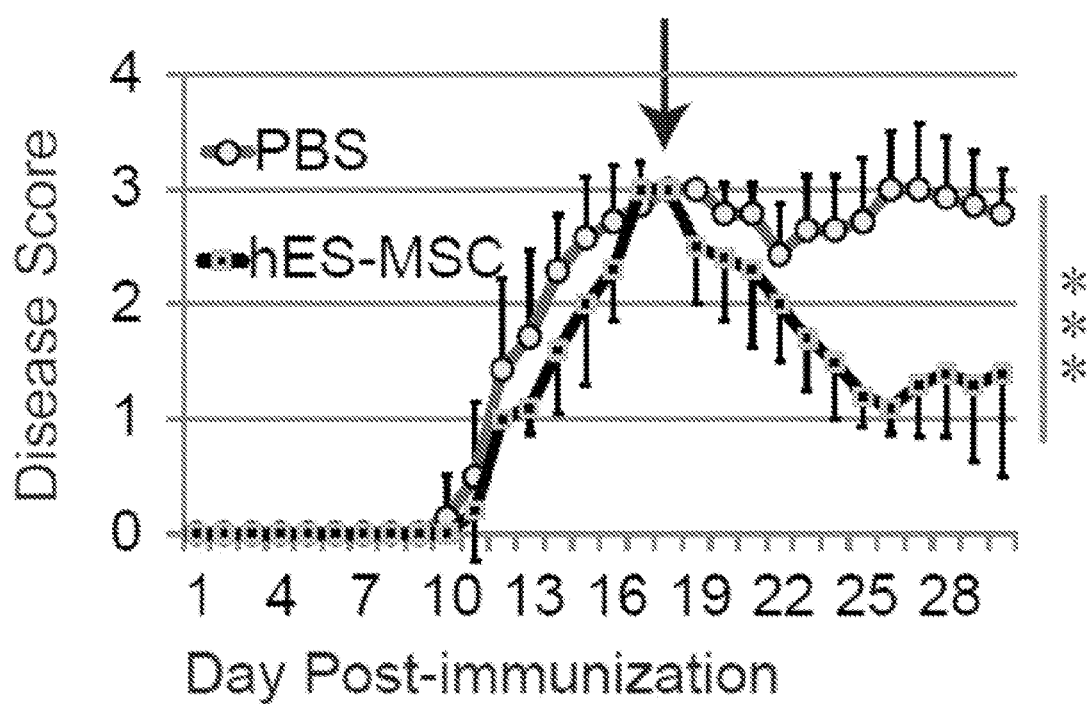
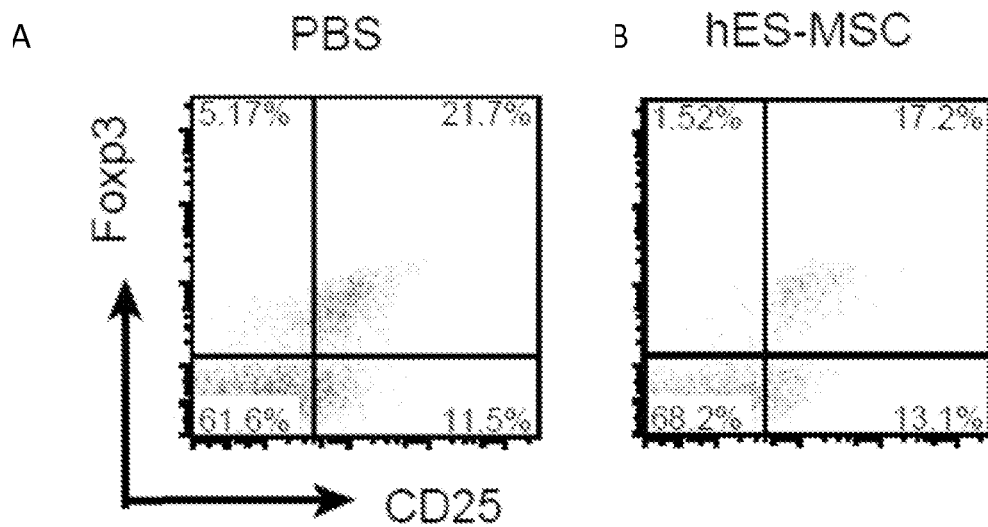
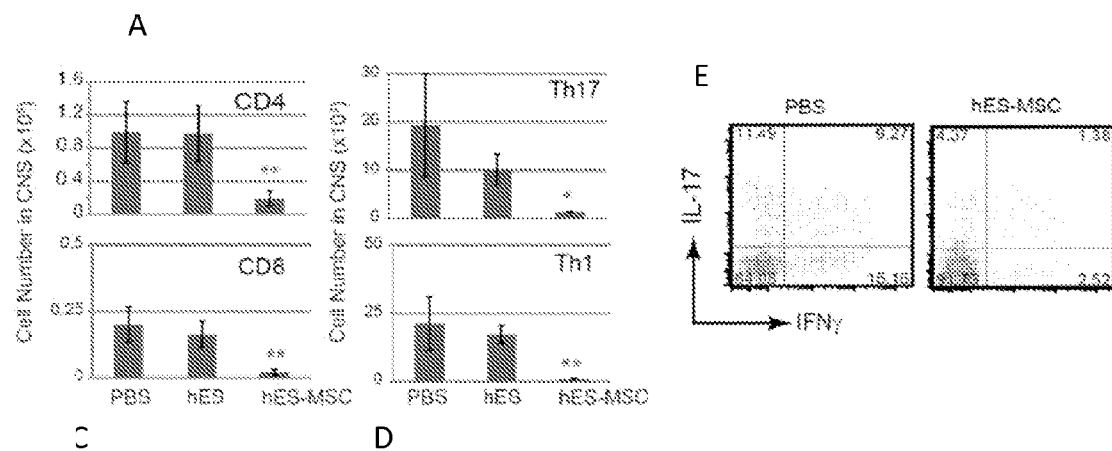


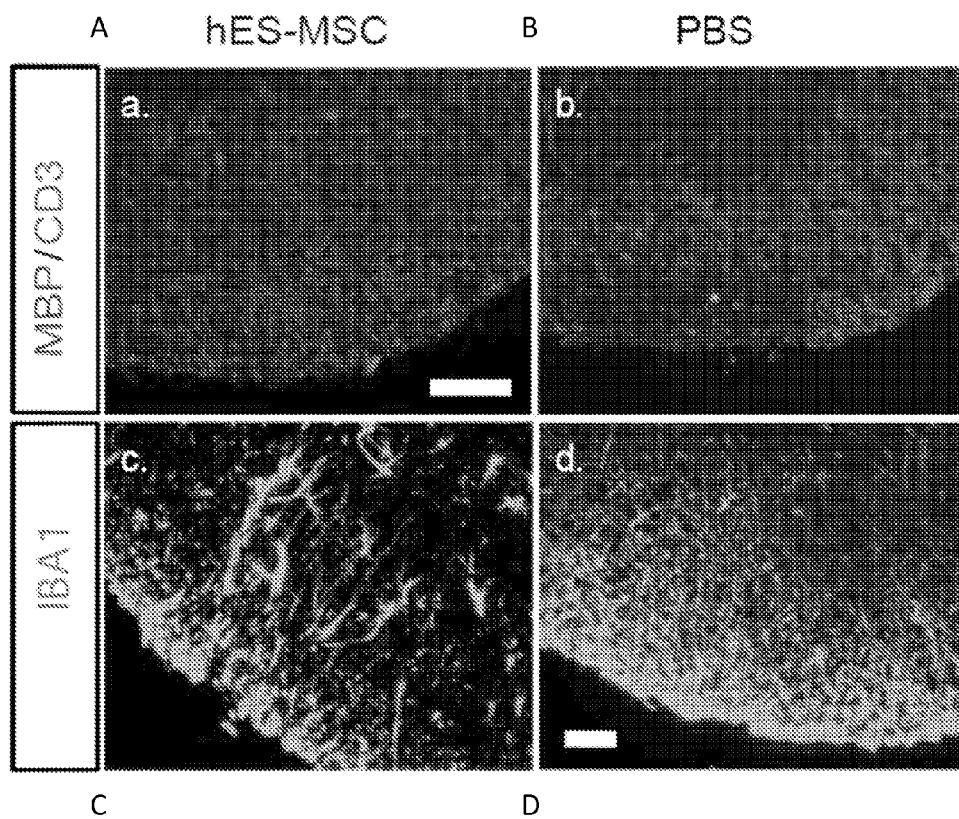
FIG. 7



FIGS. 8(A-B)



FIGS. 9 (A-F)



FIGS. 10 (A-D)

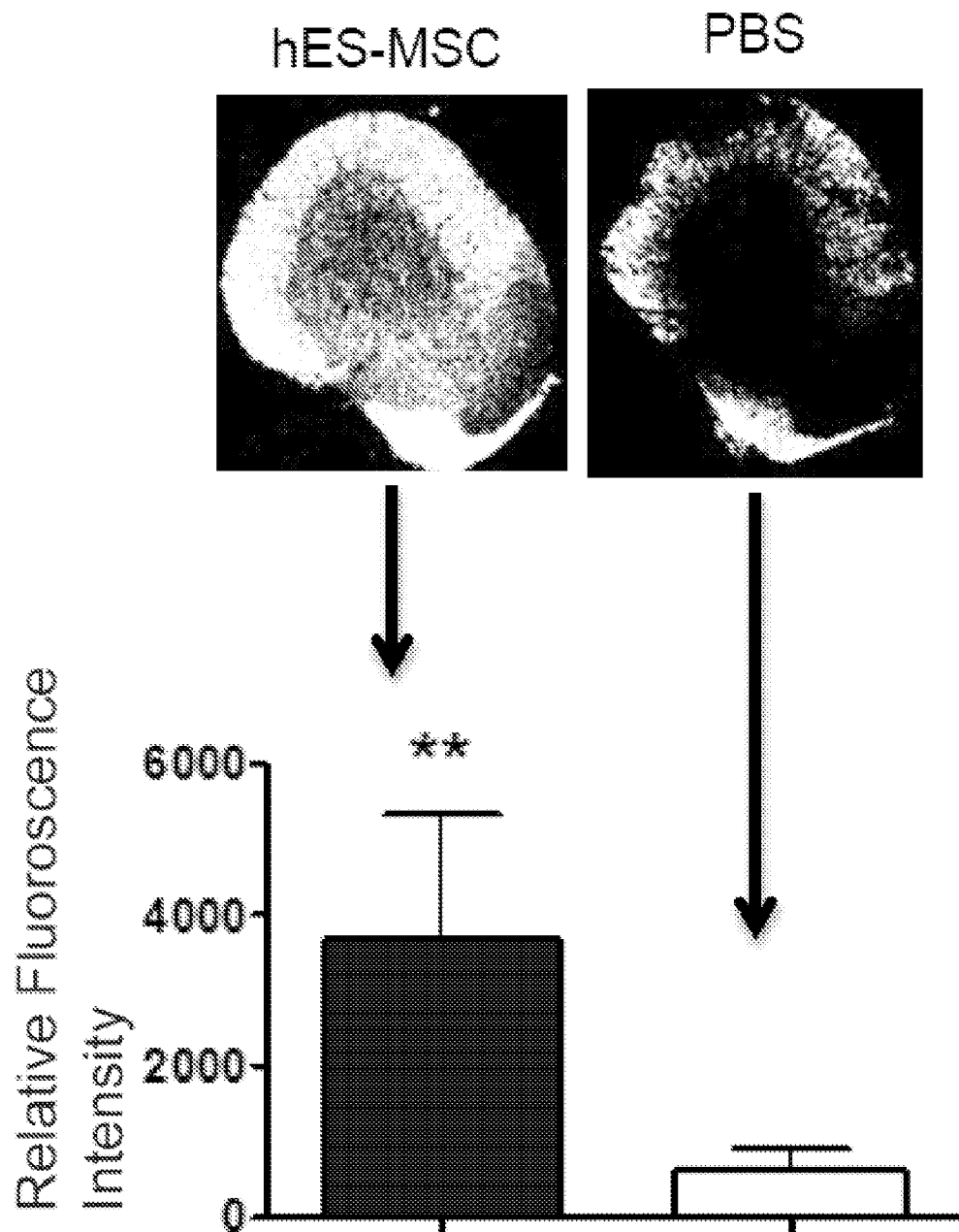
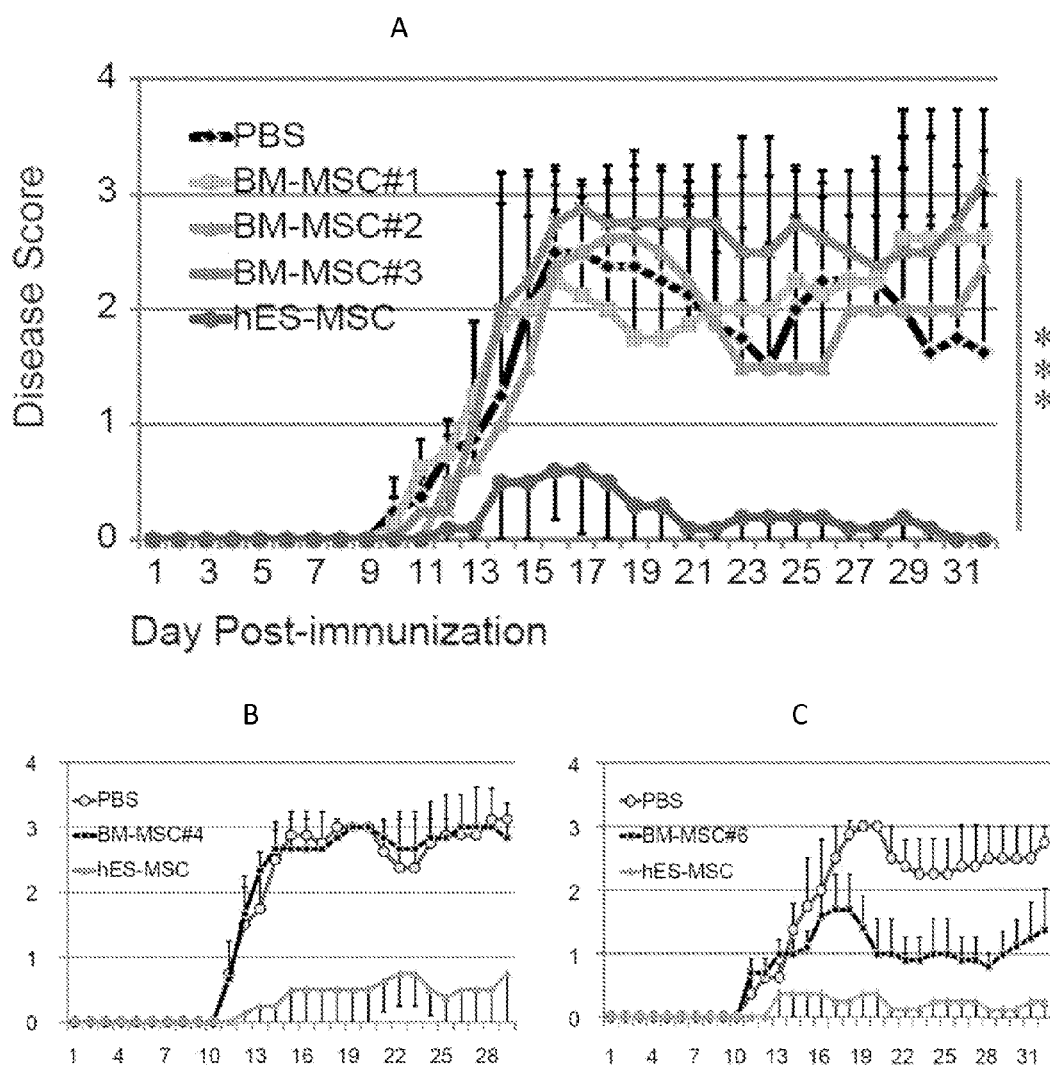
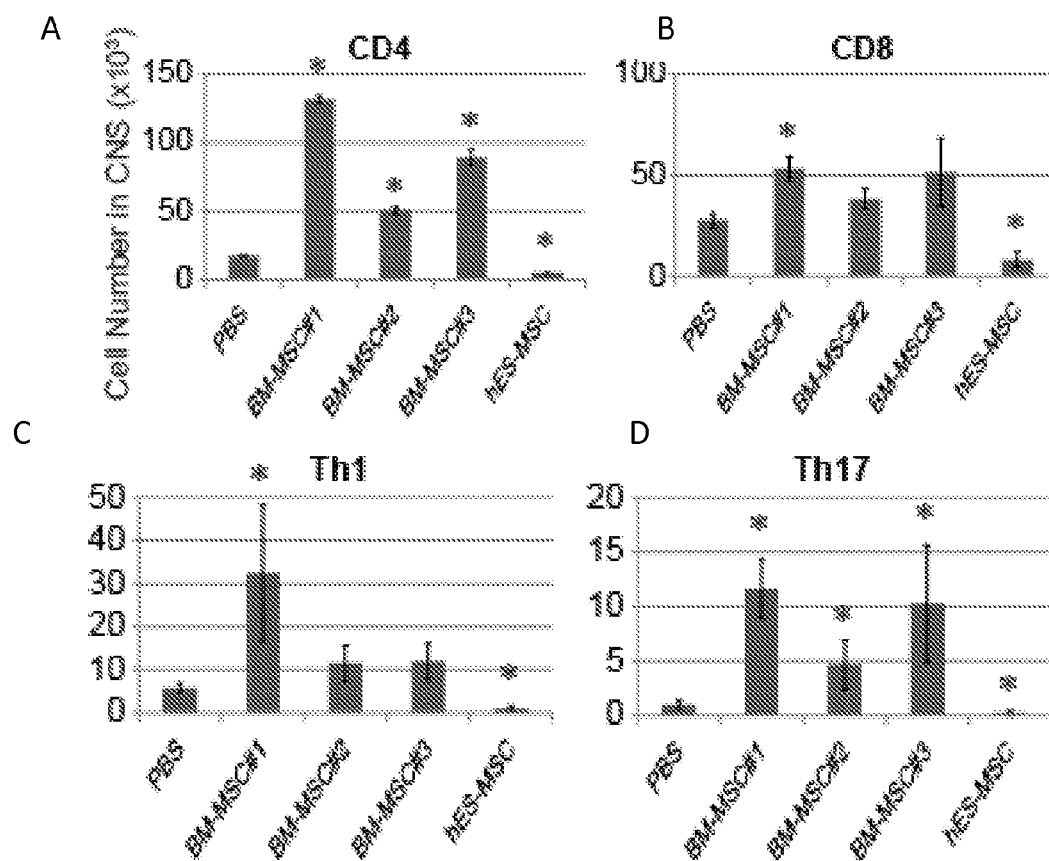


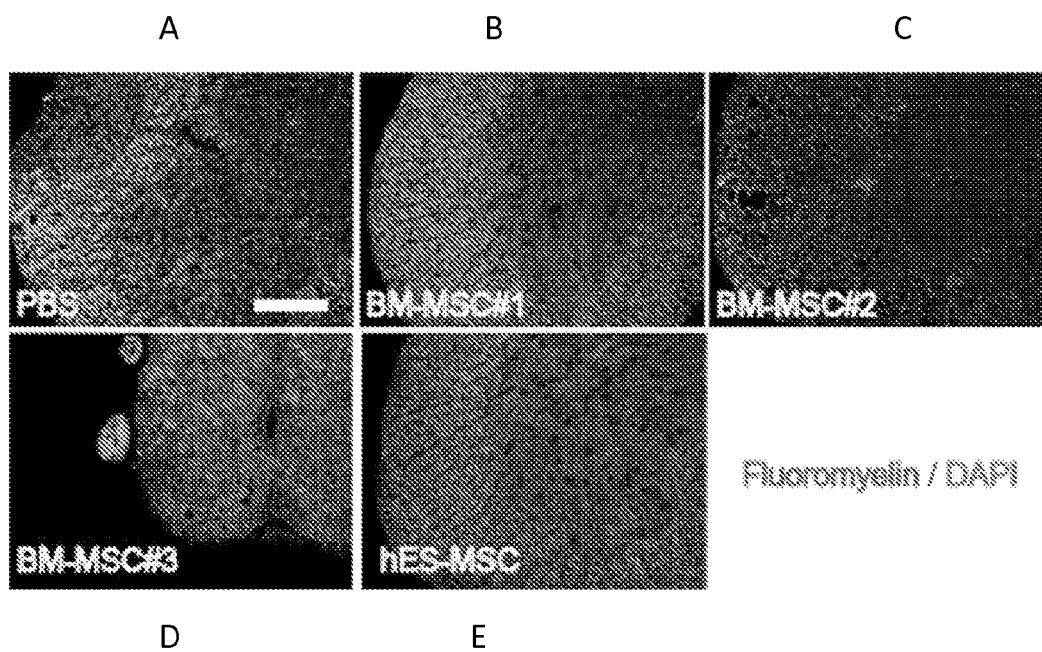
FIG. 11



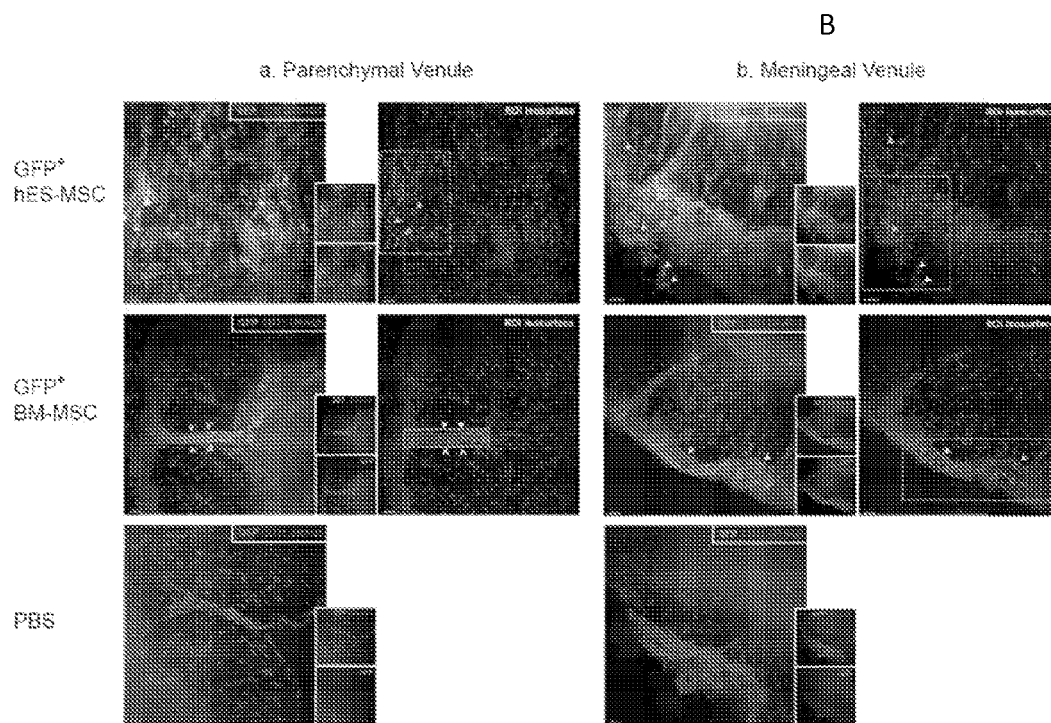
FIGS. 12 (A-C)



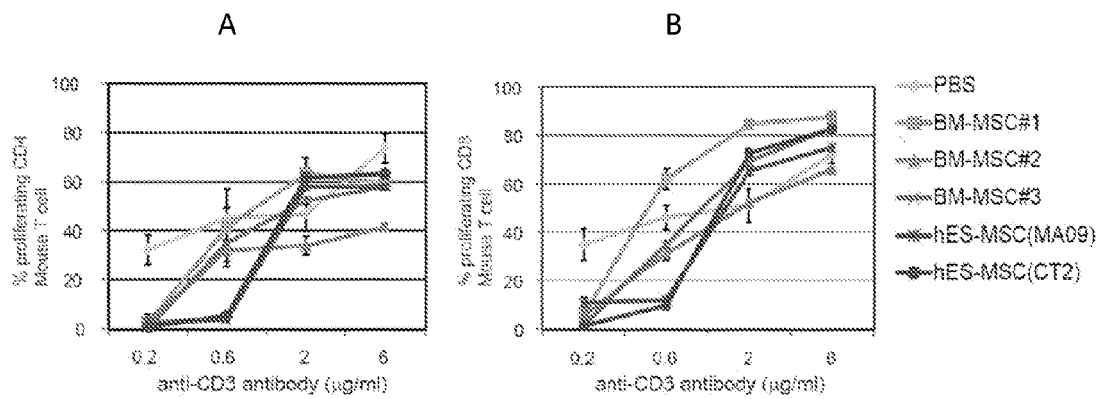
FIGS. 13 (A-D)



FIGS. 14 (A-E)



FIGS. 15 (A-B)



FIGS. 16 (A-B)

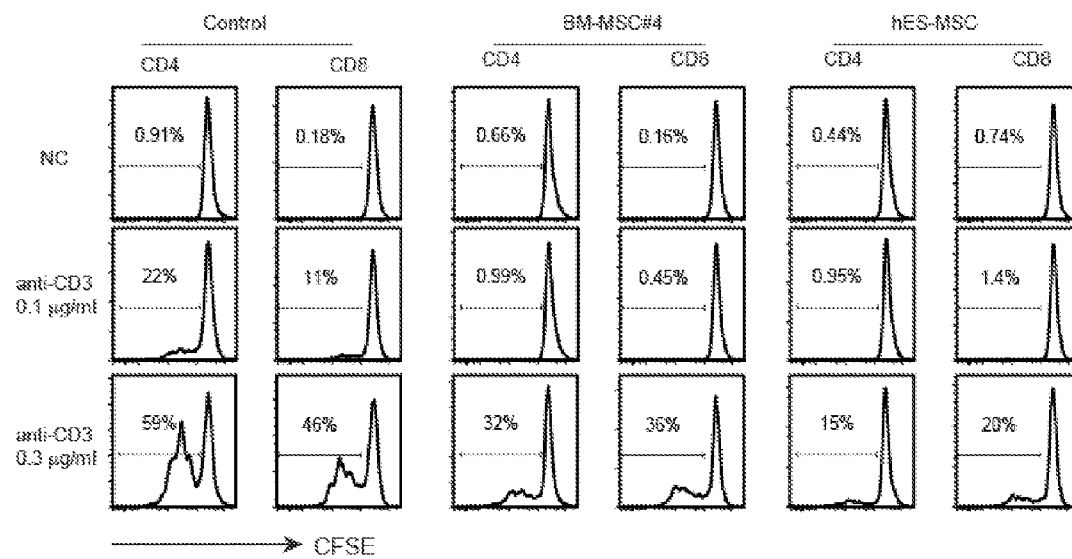
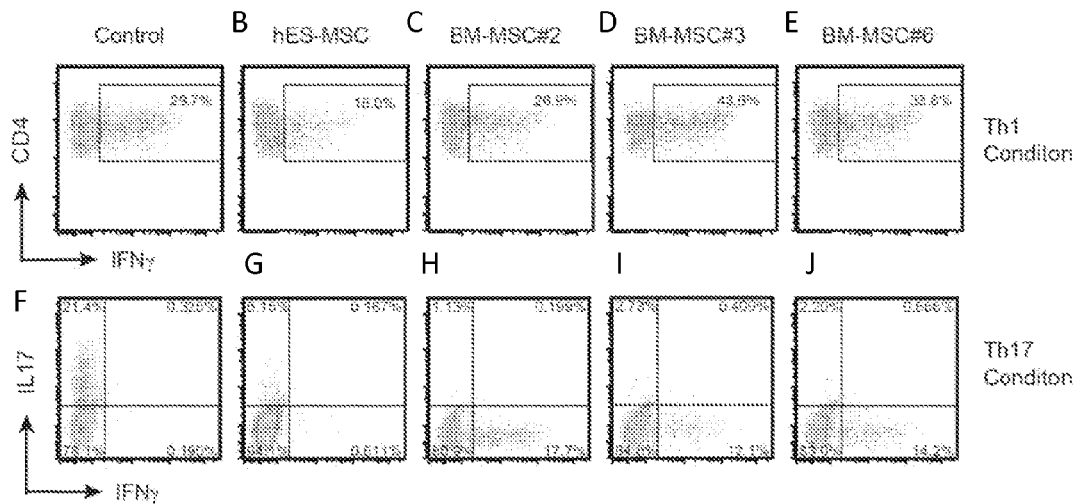
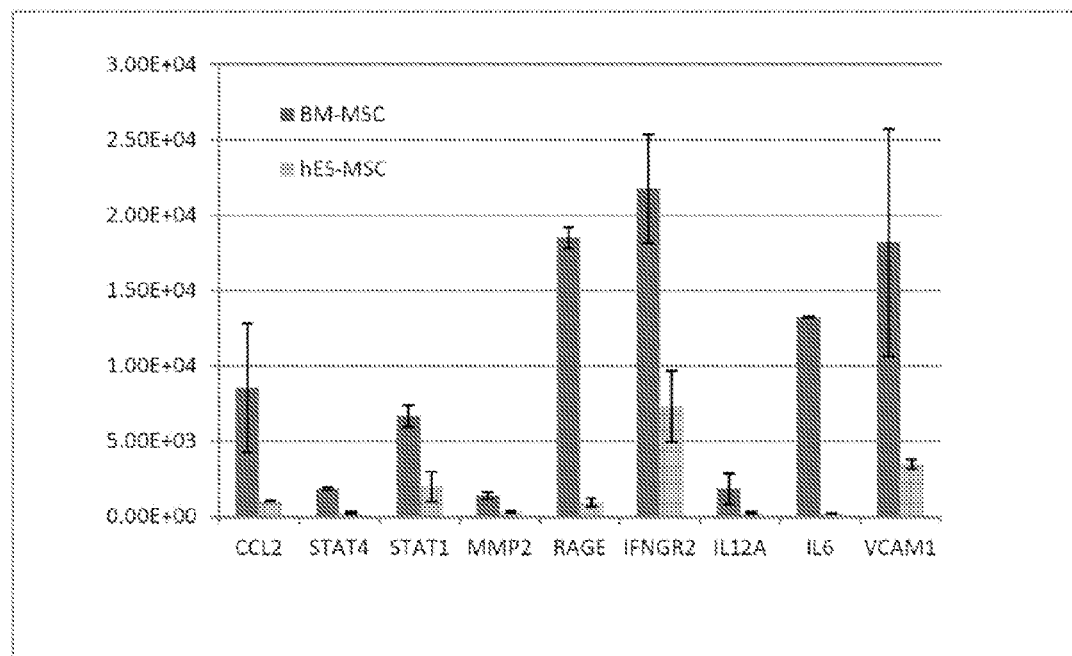
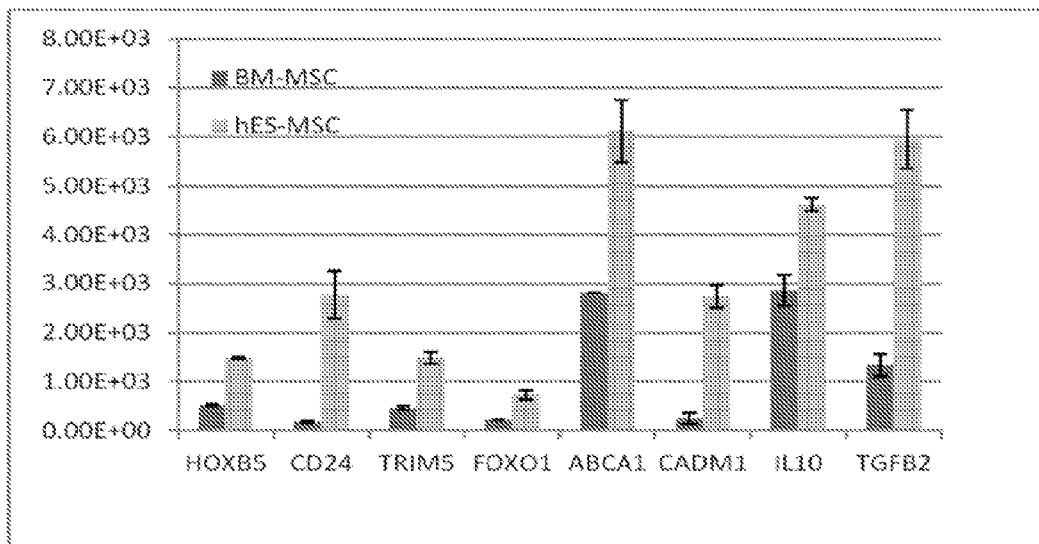


FIG. 17



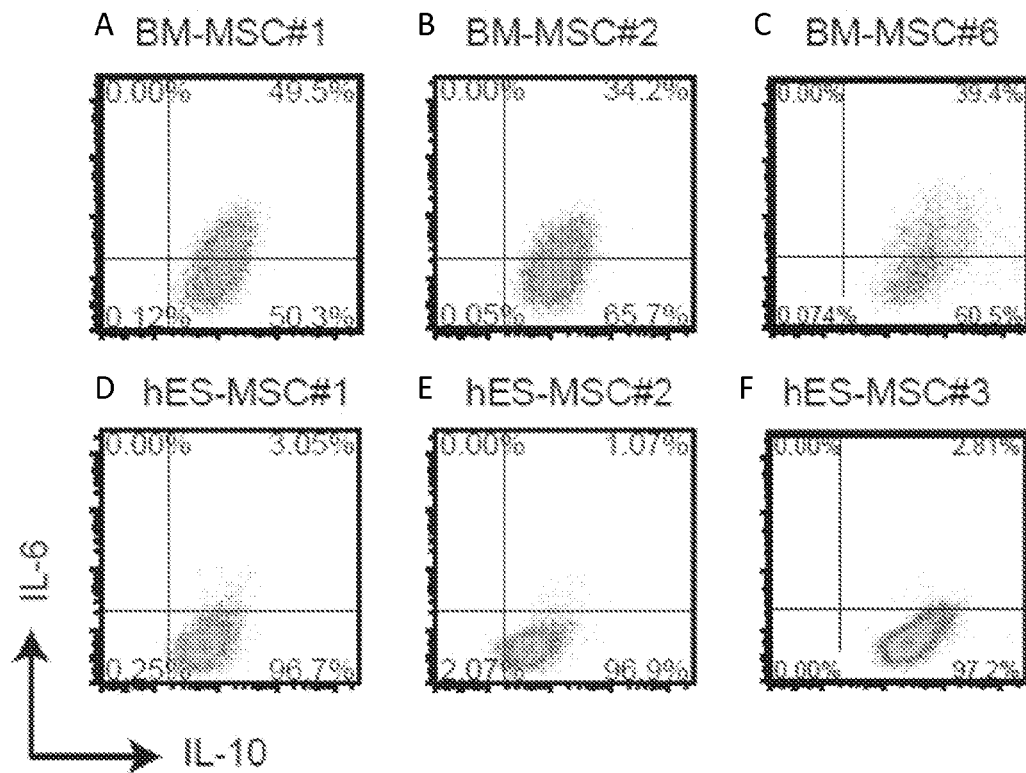
FIGS. 18 (A-J)

A

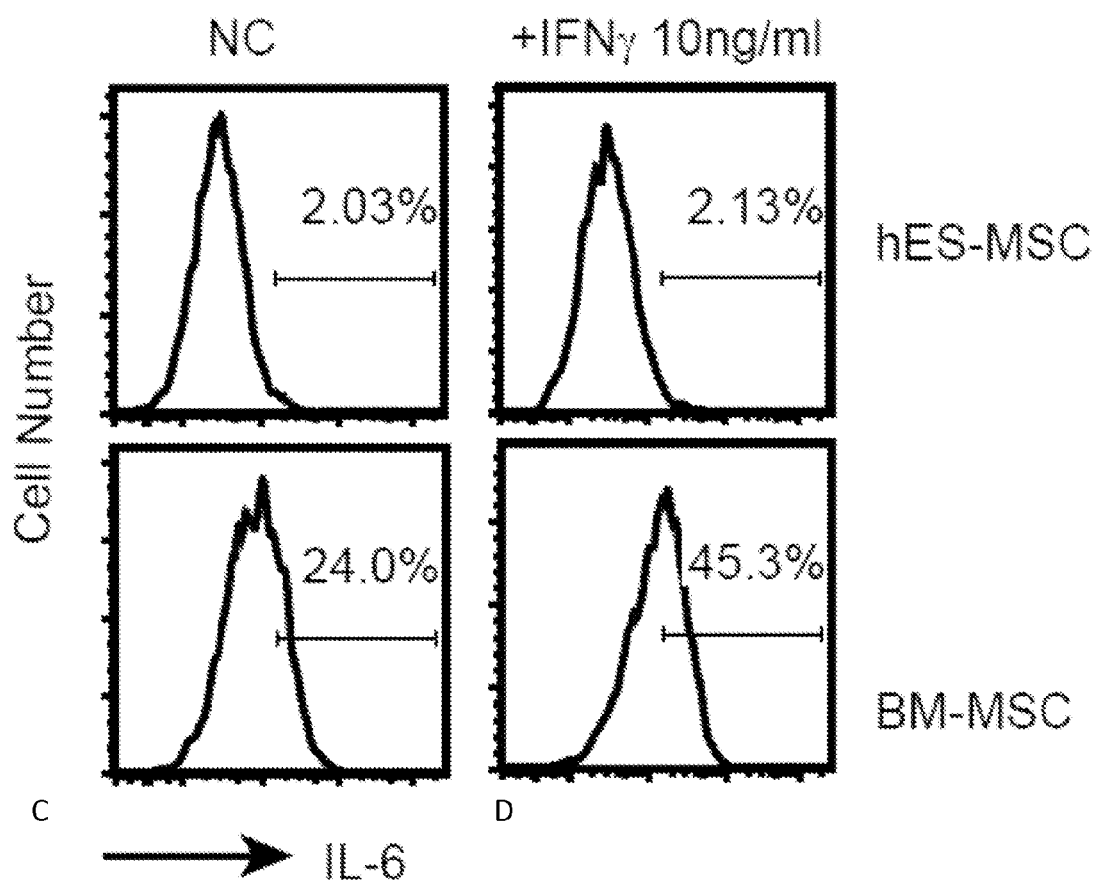


B

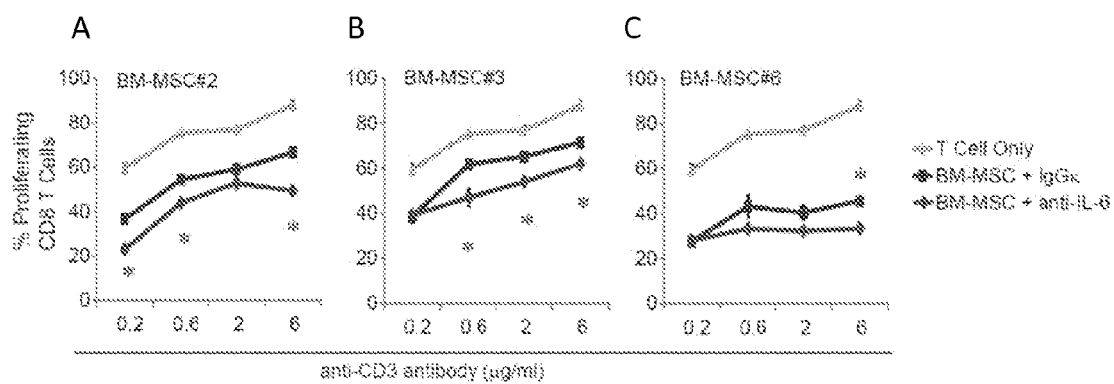
FIGS. 19 (A-B)



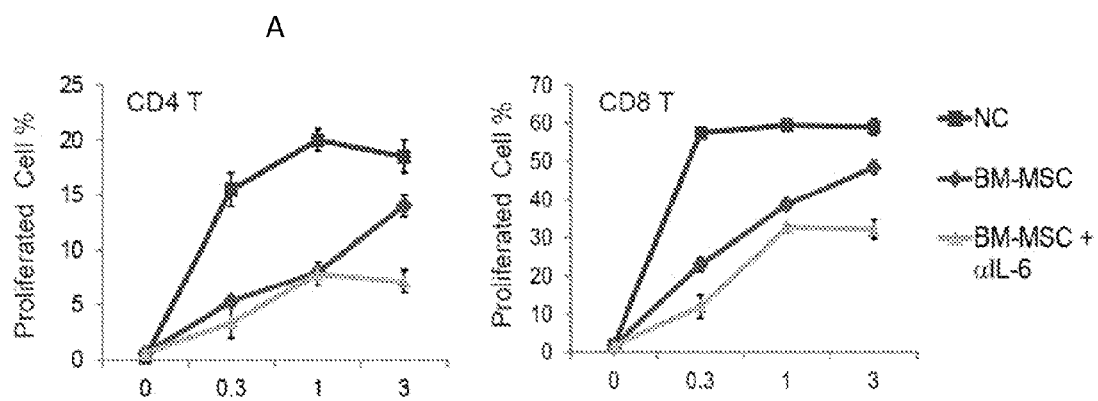
FIGS 20 (A-F)



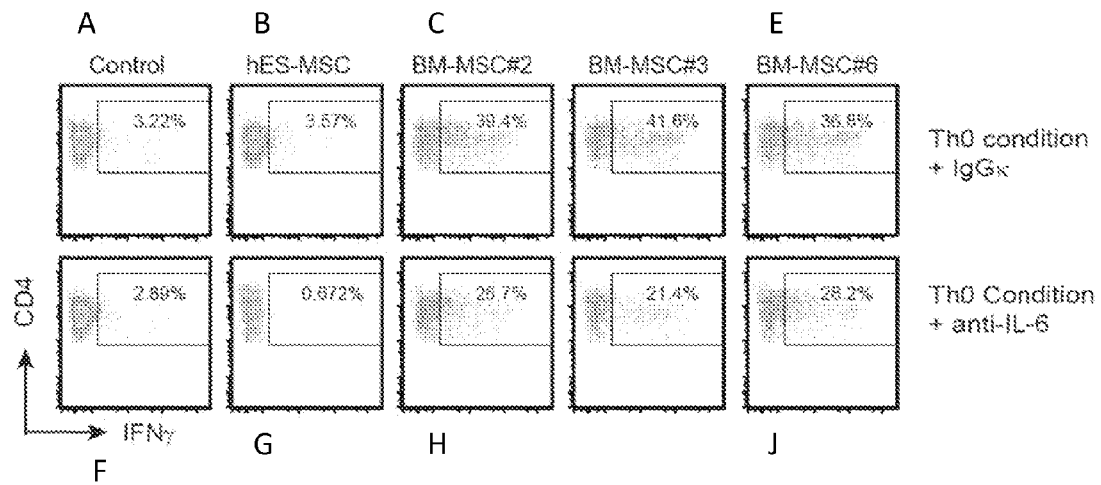
FIGS. 21 (A-D)



FIGS. 22(A-C)



FIGS. 23 (A-B)



FIGS. 24 (A-J)

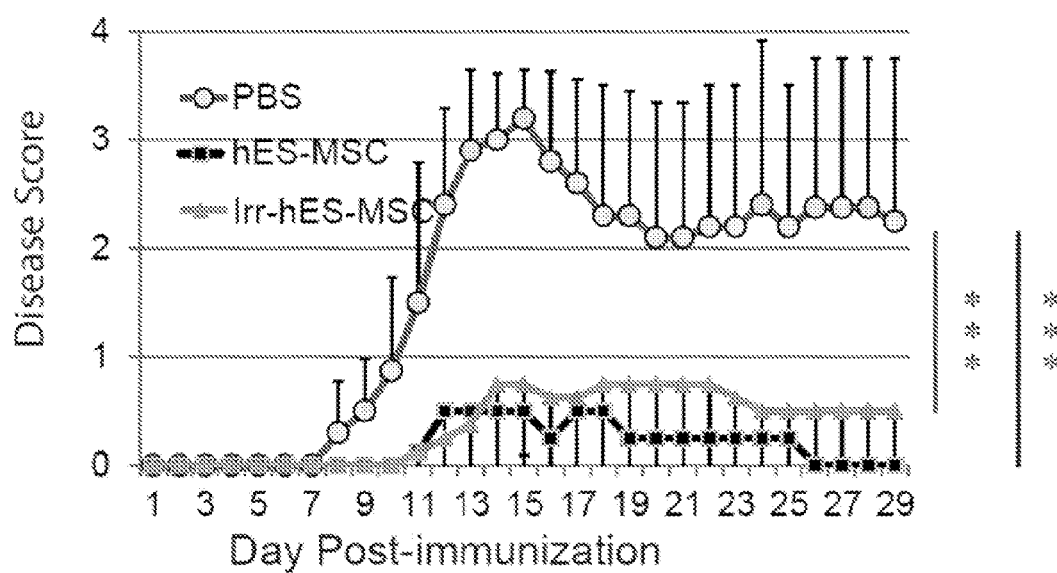


FIG. 25

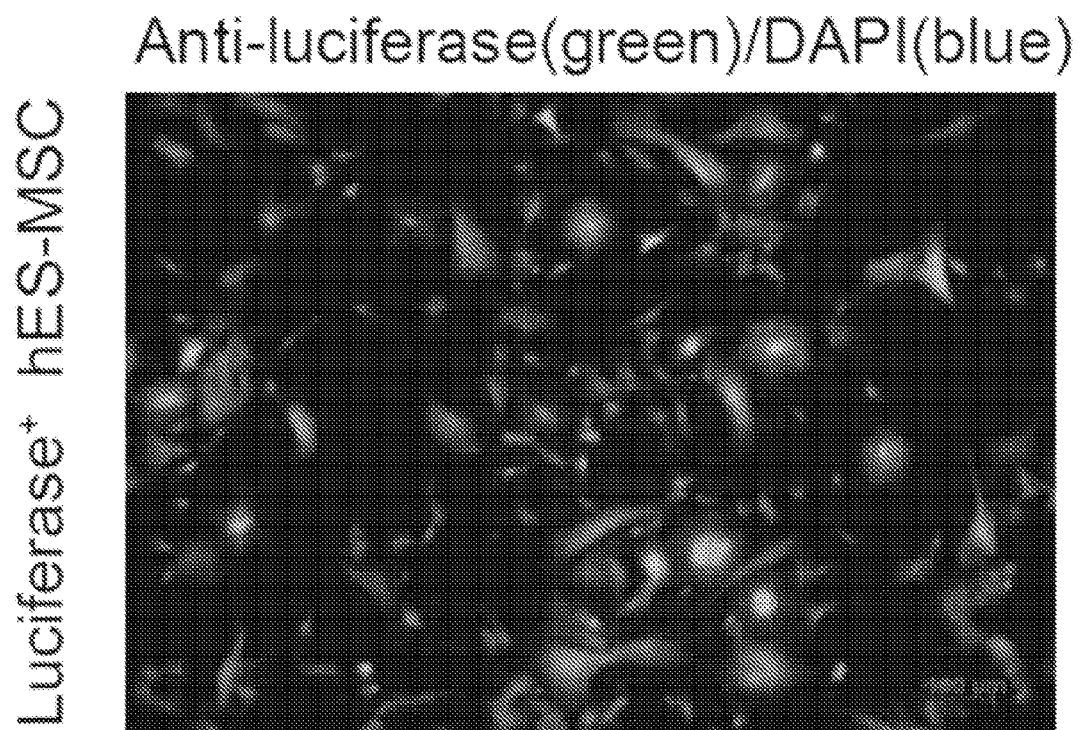
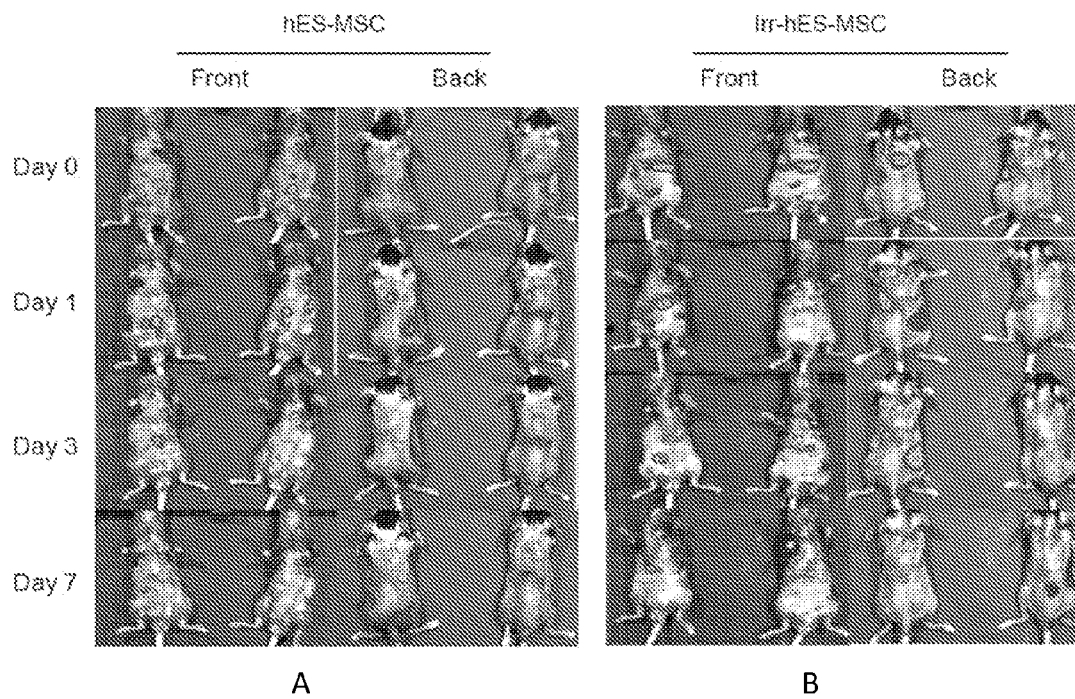


FIG. 26



FIGS. 27 (A-B)

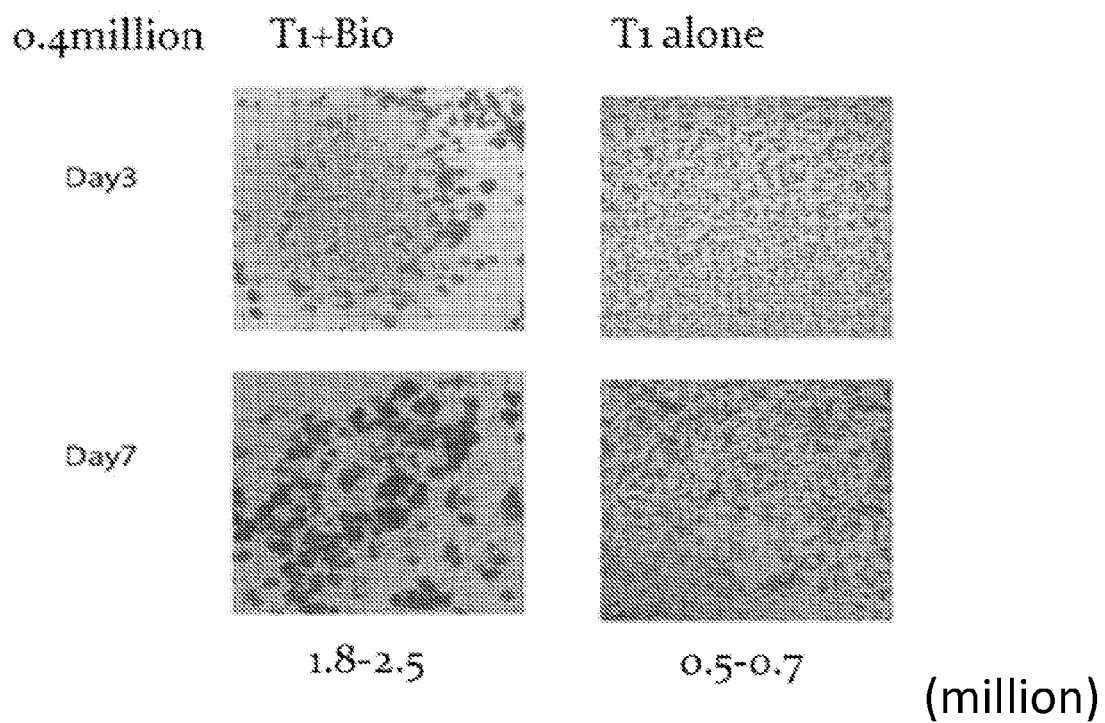


FIG. 28 (A-D)

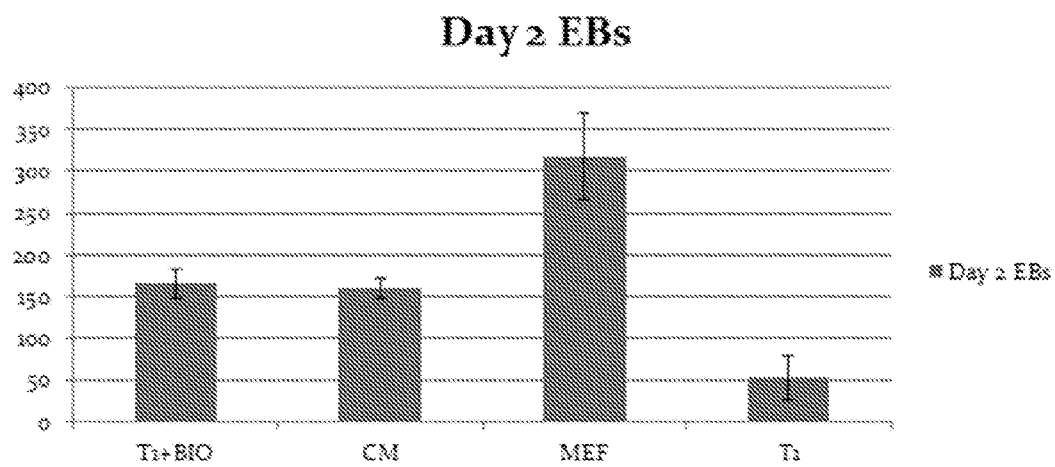


FIG. 29

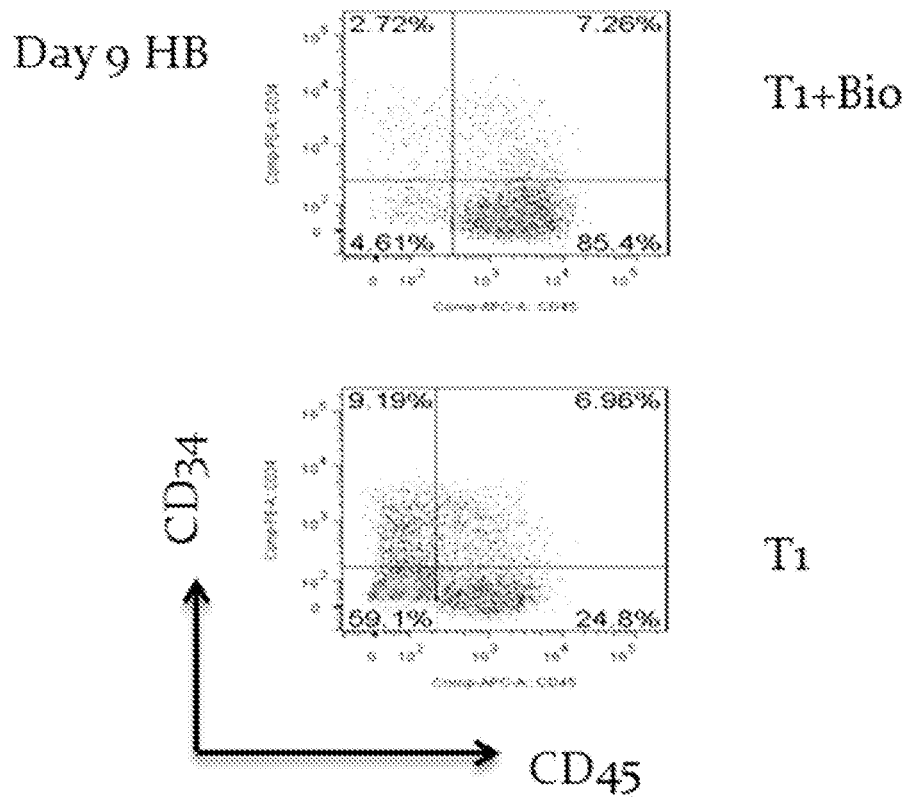


FIG. 30

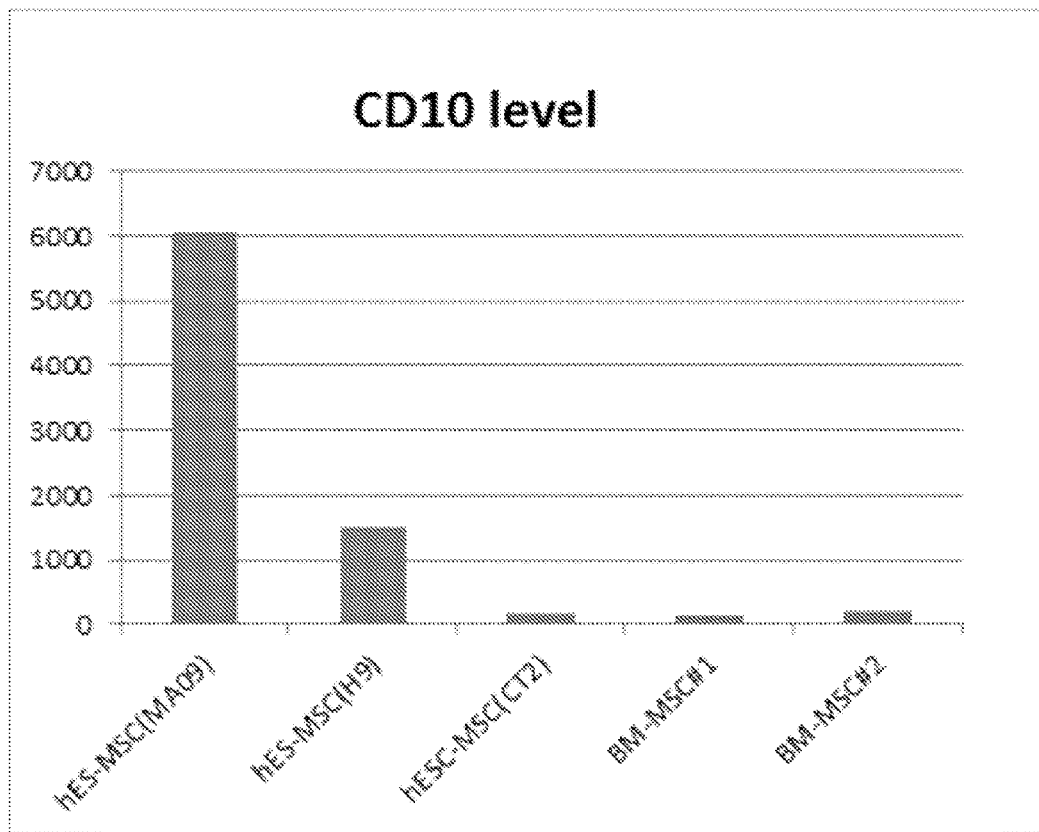


FIG. 31

US 2015/0203820 A1

Jul. 23, 2015

1

MESENCHYMAL-LIKE STEM CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS, METHODS AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority to U.S. patent application Ser. No. 61/670,787 on Jul. 12, 2012 and U.S. provisional application Ser. No. 61/762,961, filed Feb. 11, 2013, which are hereby incorporated by reference in their entireties.

1. INTRODUCTION

[0002] The present invention relates to a method of generating mesenchymal stem cells from human embryonic stem cells using a multi-step method of culturing embryonic stem cells comprising culturing embryonic stem cells under conditions sufficient to produce embryoid bodies, culturing the embryoid bodies under conditions to expand hemangio-colony forming cells in the medium comprising the embryoid bodies, and culturing the hemangio-colony forming cells under conditions that induce differentiation into mesenchymal stem cells. Also disclosed are methods of identifying highly immunosuppressive human embryonic stem cell derived mesenchymal-like stem cells. The invention also relates to the human embryonic stem-cell derived mesenchymal stem cells, solutions and pharmaceutical preparations comprising the human embryonic stem cell-derived mesenchymal stem cells, methods of using the human embryonic stem-cell derived mesenchymal stem cells for treatment and prevention of disease, specifically, T cell related autoimmune diseases, and most specifically, multiple sclerosis, and methods of using the human embryonic stem cell-derived mesenchymal stem cells for the delivery of agents across the blood brain barrier and the blood spinal cord barrier. Also provided herein are methods of using hES-MSCs to modulate the immune system, inhibit immune response to individual's self-antigen and repair damaged central nerve systems. Provided herein are compositions comprising hES-MSCs for use in immunomodulation, methods of providing modified MSC with improved immunosuppressive function through modified gene expression. Also provided are methods of using hES-MSC as drug and/or gene delivery system.

2. BACKGROUND

[0003] Multiple sclerosis (MS) is a chronic autoimmune disease caused by infiltration of peripheral immune cells into the central nervous system (CNS) through damaged blood-brain barrier (BBB) or blood-spinal cord barrier (BSCB), which causes inflammation of the myelin sheaths around neuronal axons, and causes demyelination and scarring of the axons (McFarland and Martin (2007)). Almost any neurological symptom including physical and cognitive disability can appear with MS. The incidence of the disease is approximately 0.1% worldwide, and the disease onset usually occurs in young adults (more in females) (Benito-Leon (2011)). According to the National Multiple Sclerosis Society of United States, there are more than 70 FDA-approved medications for the treatment of MS, including Avonex (IFN β -1a), Betaseron (IFN β -1b), Gilenya (a sphingosine 1-phosphate receptor modulator), Glatiramer acetate (or Copolymer 1), and Tysabri (humanized anti- α -integrin antibody). However,

these offer only palliative relief and are associated with serious adverse effects including increased infection, heart attack, stroke, progressive multifocal leukoencephalopathy, arrhythmia, pain, depression, fatigue, macula edema, and erectile dysfunction (Johnston and So (2012); Weber et al. (2012)).

[0004] Transplantation of mesenchymal stromal/stem cells (MSCs) has emerged as a potentially attractive therapy due to their immunomodulatory and neuroregenerative effects (Auletta et al., (2012); Pittenger et al. (1999)) and potential ability to repair the blood-brain barrier (Chao et al. (2009); Menge et al. (2012)). MSCs are multipotent meaning they can generate a variety of cell lineages including adipocyte, chondrocyte, and osteoblast cells. They can be derived from fetal, neonatal, and adult tissues such as the amniotic membrane, umbilical cord, bone marrow, and adipose. MSCs have several unique advantages over current pharmacotherapies, as these cells can serve as carriers of multiple and potentially synergistic therapeutic factors, and can migrate to injured tissues to exert local effects through secretion of mediators and cell-cell contact (Uccelli and Prockop (2010a)). Importantly, MSCs have been found efficacious in the treatment of mice with experimental autoimmune encephalomyelitis (EAE), a well-recognized animal model of MS (Gordon et al., 2008a; Gordon et al. (2010); Morando et al. (2012); Peron et al. (2012); Zappia et al. (2005); Zhang et al. (2005)), as well as MS patients in clinical trials (Connick et al. (2012); Karussis et al. (2010); Mohyeddin Bonab et al. (2007); Yamout et al. (2010)). Xenogeneity does not appear problematic as both mouse and human bone marrow-derived MSC (BM-MSC) can attenuate disease progression of EAE mice (Gordon et al. (2008a); Gordon et al. (2010); Morando et al. (2012); Peron et al. (2012); Zappia et al. (2005); Zhang et al. (2005)).

[0005] However, pitfalls exist for translating these findings from animals to patients. First, the limited sources and varying quality of human bone marrow (or other adult tissues) from different donors restrict the study and application of the MSCs, and prevent the standardization of the MSCs as a therapeutic product for large-scale clinical use. Second, these adult tissue-derived MSCs are highly mixed populations of cells, and perhaps only a portion of the cells exerts immunosuppressive effect. To obtain enough cells that are clinical grade for clinical use, one has to expand the MSC in vitro, which can decrease their immunosuppressive and homing abilities (Javazon et al. (2104)). Third, there are safety concerns about BM-MSC for possible malignant transformation of the cells (Wong (2011)), and potential transmission of pathogens from donors. Finally, varying effects were reported on EAE mice treated with BM-MSC in different reports (Gordon et al. (2008a); Payne et al. (2012); Zappia et al. (2005); Zhang et al. (2005)). Thus, the efficacy of BM-MSC on treatment of the disease is questionable.

[0006] Thus, there is a need for new therapies for the treatment of multiple sclerosis and other autoimmune diseases. There is also a need for an unlimited, safe, highly stable, efficient and consistent source of MSC to use as a treatment and prophylactic for these diseases as well as others.

[0007] It has been reported that human embryonic stem cells (hESC) can differentiate into embryoid bodies (EB), and then into a pool of cells with hemangioblast (HB) activities, i.e., they can further differentiate into vascular smooth muscle cells, endothelial cells, and hematopoietic cells (Chyou et al. (2008); Lu et al. (2007); Lu et al. (2009)). Therefore it was reasoned that a portion of these HB-containing cells could

US 2015/0203820 A1

Jul. 23, 2015

2

differentiate into MSCs, thus, eliminating the problems found with bone marrow-derived MSCs. These mesenchymal stem cells derived from human embryonic stem cell would be an unlimited, safe, and consistent supply of stem cells to be used to treat and prevent autoimmune diseases. Also disclosed herein are microarray analysis and other analysis, where several key factors are identified which differentially expressed in hES-MSC compared to BM-MSC.

3. SUMMARY OF THE INVENTION

[0008] The current invention is based on the surprising discovery that a portion of the HB-containing cells derived from embryonic stem cells (hES), can also differentiate into MSC, designated "hES-MSC", with high efficiency and consistency. These hES-MSCs produced from multiple hESC lines by the method of the invention, all remarkably inhibited T cell proliferation and differentiation in vitro and attenuated the disease score of EAE mice in vivo, accompanied by decreased demyelination, T cell infiltration, and microglial responses in the CNS. In contrast, BM-MSC from multiple sources had no effect at all on the EAE mice although they may reduce T cell proliferation and differentiation in vitro.

[0009] Thus, the present invention overcomes the problems described above by providing a method of generating mesenchymal stem cells (MSC) in vitro from human embryonic stem cells. The ability to generate the hES-MSC by the novel method disclosed herein allows the production of cells that can be used in a variety of therapeutic applications, including the treatment and prevention of multiple sclerosis, and other autoimmune diseases. Additionally, the hES-MSC produced by the novel method have the ability to cross the brain-blood barrier (BBB) and the blood-spinal cord barrier (BSCB) allowing them to be used for a variety of therapeutic applications, including drug delivery. The methods of the invention provide further utility in that they enable the generation of large numbers of hES-MSC that can be used at commercial scale.

[0010] Additionally, the present invention includes the human embryonic-derived mesenchymal stem cells produced by this method.

[0011] One embodiment of the present invention is a method for generating and expanding human embryonic-mesenchymal stem cells in vitro, said method comprising the steps of:

[0012] a. culturing a cell culture comprising human embryonic stem cells in serum-free medium in the presence of at least one growth factor in an amount sufficient to induce the differentiation of said embryonic stem cells to differentiate into embryoid bodies;

[0013] b. disaggregating the embryoid bodies into single cells and adding at least one growth factor to said culture comprising single cells from embryoid bodies and continuing to culture in serum-free medium, wherein said growth factor is in an amount sufficient to expand human hemangio-colony forming cells in said hemangio-colony culture;

[0014] c. disaggregating the hemangio-colony forming cells into single cells;

[0015] d. culturing the single cells in serum-containing media or serum-free media in an amount sufficient to induce the differentiation of said hemangio-colony forming single cells into mesenchymal stem cells,

[0016] In certain embodiments, at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the resulting human embryonic-mesenchymal stem cells express CD73.

[0017] In certain embodiments, at least about 95% of the resulting human embryonic-mesenchymal stem cells express CD73. In certain embodiments, more than 95% of the resulting human embryonic-mesenchymal stem cells express CD73.

[0018] In certain embodiments, the medium contains growth factor including vascular endothelial growth factor (VEGF), bone morphogenic protein (BMP), stem cell factor (SCF), Flt-3L (FL), thrombopoietin (TPO), erythropoietin (EPO) or a combination thereof.

[0019] In certain embodiments, the serum-containing medium contains fetal calf serum, L-gultamine and the serum-free medium contains knockout serum replacement (KOSR) or bovine serum albumin (BSA).

[0020] In certain embodiments, there is an additional step of irradiating the resulting human embryonic-mesenchymal stem cells with gamma radiation ranging from 1 gy to 200 gy.

[0021] In certain embodiments, the method for generating and expanding human embryonic-mesenchymal stem cells results in at least 10,000 human embryonic-mesenchymal stem cells, at least 50,000 human embryonic-mesenchymal stem cells, at least 100,000 human embryonic-mesenchymal stem cells, at least 500,000 human embryonic-mesenchymal stem cells, at least 1×10^6 human embryonic-mesenchymal stem cells, at least 5×10^6 human embryonic-mesenchymal stem cells, at least 1×10^7 human embryonic-mesenchymal stem cells, at least 5×10^7 human embryonic-mesenchymal stem cells, at least 1×10^8 human embryonic-mesenchymal stem cells, at least 5×10^8 human embryonic-mesenchymal stem cells, at least 1×10^9 human embryonic-mesenchymal stem cells, at least 5×10^9 human embryonic-mesenchymal stem cells, or at least 1×10^{10} human embryonic-mesenchymal stem cells. These methods result in cell solutions that may comprise between 10,000 and 10 billion human embryonic-mesenchymal stem cells. In certain embodiments, at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the resulting human embryonic-mesenchymal stem cells express one or more hES-MSC differential markers. In certain embodiment, the marker is CD73, CD90 and CD105.

[0022] In one embodiment, the hES-MSCs remarkably attenuate the disease score of the EAE mice, accompanied by decreased demyelination. T cell infiltration, and microglial responses. In addition, the hES-MSCs have much stronger immunosuppressive activity in vivo and in vitro when compared to bone marrow derived MSCs (BM-MSC). Also provided herein are key proteins/molecules that are differentially expressed between hES-MSC and BM-MSCs. Provided herein are methods of identifying hES-MSCs with improved immunosuppressive activity by measuring the expression level of the protein/molecular markers. Also disclosed are methods of genetic modification to improve immunosuppressive activity of hES-MSCs.

[0023] A further embodiment of the present invention is a solution comprising human embryonic-mesenchymal stem cells comprising at least 10,000 human embryonic-mesenchymal stem cells, at least 50,000 human embryonic-mesenchymal stem cells, at least 100,000 human embryonic-mesenchymal stem cells, at least 500,000 human embryonic-mesenchymal stem cells, at least 1×10^6 human embryonic-

US 2015/0203820 A1

Jul. 23, 2015

3

mesenchymal stem cells, at least 5×10^6 human embryonic-mesenchymal stem cells, at least 1×10^7 human embryonic-mesenchymal stem cells, at least 5×10^7 human embryonic-mesenchymal stem cells, at least 1×10^8 human embryonic-mesenchymal stem cells, at least 5×10^8 human embryonic-mesenchymal stem cells, at least 1×10^9 human embryonic-mesenchymal stem cells, at least 5×10^9 human embryonic-mesenchymal stem cells, or at least 1×10^{10} human embryonic-mesenchymal stem cells.

[0024] In certain embodiments, the culture volume is from 2 ml for at least 10,000 cells, 10 ml for at least 100,000 cells, 100 ml for at least 1,000,000 cells, 1000 ml for at least 10,000,000 cells, and up to 4000 ml of media for 5×10^5 cell.

[0025] These solutions can be injected into a subject. These solutions can be frozen. These solutions can be used for the manufacture of a medicament for a disease that can be treated by the administration of human embryonic-mesenchymal stem cells.

[0026] This invention also provides a method for producing a solution of human embryonic-mesenchymal stem cells suitable for injection into a patient comprising the steps of isolating the solution of cells described in the preceding paragraph and placing the cells into solution suitable for injection into a patient. This invention also provides a method of producing a solution of human embryonic-mesenchymal stem cells suitable for freezing comprising the steps of isolating the cells described in the preceding paragraph and placing into a solution suitable for freezing.

[0027] Yet another embodiment of the present invention is a human embryonic-mesenchymal stem cell expressing one or more of cell marker proteins including CD73, CD90, CD105, CD13, CD29, CD54, CD44 or a combination thereof. In a further embodiment, the human embryonic-mesenchymal stem cell does not express or expresses low levels of one or more cell marker proteins including CD34, CD31, CD45 or a combination thereof. In a further embodiment, the human embryonic-mesenchymal stem cell does not express or expresses low levels of one or more pro-inflammatory proteins including MMP2, RAGE, IFN γ R1, IFN γ R2, IL-12, TNF α , IL-6, VCAM1 or a combination thereof. In certain embodiments, the human embryonic-mesenchymal stem cell expressed at least half of the level of the above markers as compared to Bone marrow derived MSC.

[0028] A further embodiment of the present invention is a cell culture comprising human embryonic-mesenchymal stem cells expressing one or more of cell marker proteins including CD73, CD90, CD105, CD13, CD29, CD54, and CD44. In a further embodiment, the human embryonic-mesenchymal stem cells in the cell culture do not express or express low levels of one or more cell marker proteins including CD34, CD31 and CD45. In a further embodiment, the human embryonic-mesenchymal stem cells in the cell culture do not express or express low levels of one or more pro-inflammatory proteins including MMP2, RAGE, IFN γ R1, IFN γ R2, IL-12, TNF α , IL-6, and VCAM1.

[0029] In certain embodiments, the cell culture comprises at least 1×10^6 human embryonic-mesenchymal stem cells, at least 1×10^7 human embryonic-mesenchymal stem cells at least 1×10^8 human embryonic-mesenchymal stem cells, at least 1×10^9 human embryonic-mesenchymal stem cells, or at least 1×10^{10} human embryonic-mesenchymal stem cells. For 1×10^6 cell, the initial cell culture volume will be 10-20 ml.

[0030] In further embodiments, at least about 90% of the human embryonic-mesenchymal stem cells in the cell culture

express the CD73 protein, at least more than 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the human embryonic-mesenchymal stem cells express the CD73 protein.

[0031] In further embodiments, at least about 75%, 80%, 85%, 90%, 95%, 99% of the human embryonic-mesenchymal stem cells in the cell culture express at least one cell marker protein selected from the group consisting of CD90, CD105, CD44, and CD29.

[0032] In further embodiments, at least about 80%, 85%, 90%, 95%, 99% of the human embryonic-mesenchymal stem cells in the cell culture do not express or express low levels of at least one cell marker including CD34, CD31, and CD45.

[0033] In further embodiments, at least about 75%, 80%, 85% 90%, 90%, 95%, 99% of the human embryonic-mesenchymal stem cells in the cell culture do not express or express low levels of at least one pro-inflammatory protein including MMP2, RAGE, IFN γ R1, IFN γ R2, IL-12, TNF α , IL-6, and VCAM1. In certain embodiments, the hES-MSC express high levels of CD24, TGF β 2 or both.

[0034] In certain embodiments, the human embryonic-mesenchymal stem cells or cell cultures described in the preceding paragraphs are irradiated using gamma radiation.

[0035] Provided herein are pharmaceutical preparations comprising any one of the human embryonic-mesenchymal stem cells or cell cultures described in the preceding paragraphs and pharmaceutically acceptable carriers.

[0036] Provided herein are cryopreserved preparations of any of the human embryonic-mesenchymal stem cells or cell cultures described in the preceding paragraphs.

[0037] Provided herein are methods of treating or preventing a T cell related autoimmune disease in a subject in need thereof, comprising the steps of administering a therapeutically effective amount of solution, cell culture or pharmaceutical preparation comprising human embryonic-mesenchymal stem cells as described in the preceding paragraphs, to the subject in need thereof. The T cell related autoimmune diseases include but are not limited to Crohn's disease, inflammatory bowel disease, graft versus host disease, systemic lupus erythematosus, and rheumatoid arthritis, T cell mediated delayed type hypersensitivity (Type IV hypersensitivity) i.e. Type I diabetes mellitus, MS, RA, Hashimoto's thyroiditis, Crohn's, contact dermatitis, *Scleroderma*, etc.

[0038] In certain embodiments, the subject is preferably a mammal or avian, and most preferably human. In certain embodiments, the solution, cell culture or pharmaceutical preparation comprises irradiated or non-irradiated human embryonic-mesenchymal stem cells. In certain embodiments, the method for treating or preventing disease includes combination therapy with one or more therapeutic agents for the treatment or prevention of disease.

[0039] In certain embodiments, the methods for treating or preventing multiple sclerosis disease in a subject in need thereof, comprise the steps of administering a therapeutically effective amount of solution, cell culture or pharmaceutical preparation comprising human embryonic-mesenchymal stem cells as described in the preceding paragraphs, to the subject in need thereof. Multiple sclerosis can be relapsing/remitting multiple sclerosis, progressive/relapsing multiple sclerosis, primary multiple sclerosis, or secondary multiple sclerosis. The subject is preferably a mammal or avian, and most preferably human. The solution, cell culture or pharmaceutical preparation can comprise irradiated or non-irradiated human embryonic-mesenchymal stem cells.

US 2015/0203820 A1

Jul. 23, 2015

4

[0040] The method further comprises the administration of additional therapeutic agents to the subject, including but not limited to, fingolimod, adrenocorticotrophic hormone (ACTH), methylprednisolone, dexamethasone, IFN β -1a, IFN-1b, gliatrimmer acetate, cyclophosphamide, methotrexate, azathioprine, cladribine, cyclosporine, mitoxantrone, and sulfasalazine. In yet another embodiment, one or more of these therapeutic agents can be attached to the hES-MS-C in order to cross the blood-brain and/or blood-spinal cord barrier, for delivery of the therapeutic agent to the central nervous system.

[0041] Provided herein is a method of delivering an agent through the blood-brain barrier and/or the blood-spinal cord barrier, said method comprising the steps of: attaching or conjugating the agent to a human embryonic-mesenchymal stem cell to form a complex; and administering the human embryonic-mesenchymal stem cell-agent complex to a subject in need thereof, wherein the human embryonic-mesenchymal stem cell is capable of crossing the blood-brain barrier and/or the blood-spinal cord barrier and the agent is for the treatment, prevention or diagnosis of a disease or injury in the subject in need thereof. The human embryonic-mesenchymal stem cells may be in the form of a single cell, a cell culture, a solution or a pharmaceutical preparation. Agent would include but are not limited to drugs, proteins, DNA, RNA, and small molecules.

[0042] Provided herein is a method of selecting clinical grade hES-MS-C for the treatment of autoimmune diseases, said hES-MS-C having the following characteristics: (i) contain >95% of cells expressing group-1 markers; (ii) contain >80% of cells expressing group 2 markers; (iii) contain <5% of cells expressing group-3 markers (iv) expressing IL-10 and TGF β ; (v) contain <2% of cells expressing IL-6, IL-12 and TNF α ; and (vi) contains <0.0010% of cells co-expressing all group-4 markers, wherein group-1 markers are CD73, CD90, CD105, CD146, CD166, and CD44, group-2 markers are CD13, CD29, CD54, CD49E, group-3 markers are CD45, CD34, CD31 and SSEA4, and group-4 markers are OCT4, NANOG, TRA-1-60 and SSEA4.

[0043] Provided herein is a method of modifying mesenchymal stem cells to produce a population of modified MSC having the following characteristics: (i) contain >95% of cells expressing group-1 markers, (ii) contain >80% of cells expressing group 2 markers; (iii) contain <5% of cells expressing group-3 markers (iv) expressing IL-10 and TGF β ; (v) contain <2% of cells expressing IL-6, IL-12 and TNF α ; and (vi) contains <0.001% of cells co-expressing all group-4 markers, wherein group-1 markers are CD73, CD90, CD105, CD146, CD166, and CD44, group-2 markers are CD13, CD29, CD54, CD49E, group-3 markers are CD45, CD34, CD31 and SSEA4, and group-4 markers are OCT4, NANOG, TRA-1-60 and SSEA4. Provided herein is a method of modifying MSC to produce a MSC with specific biomarker profile. Similar to hES-MS-C, the modified MSC are useful for treatment of various diseases as listed in Section 5.14.

[0044] Provided herein are conditioned medium, concentrate of conditioned medium cell lysate or other derivatives thereof that comprises one or more biomolecules secreted by the MSC as described. Provided herein is a method of using MSC as described herein as feeder cells for bone marrow hematopoietic stem cell expansion and umbilical-cord hematopoietic stem cell expansion. In certain embodiments, the MSC suitable for the disclosed method express Stro3. In certain embodiment, MSC is co-cultured with bone marrow

hematopoietic stem cells and/or umbilical-cord hematopoietic stem cells. In certain embodiment, the MSC is mesenchymal stromal cells. Provided herein is a co-culture of MSC as described herein and bone marrow hematopoietic stem cells. Provided herein is a co-culture of MSC as described herein and umbilical-cord hematopoietic stem cells.

[0045] Also disclosed are kits comprising MSC described herein. In certain embodiments, the kits comprise hES-MS-C and a cell delivery carrier.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[0046] For the purpose of illustrating the invention, there are depicted in drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

[0047] FIGS. 1A-D show a phase contrast images of hESC (CT2) differentiation into MSCs through multiple stages. (hESC: human embryonic stem cells; EB: embryo body; HB-enriched: hemangioblast-enriched; hES-MS-C: hES cell derived mesenchymal stem cell)

[0048] FIGS. 2A-C depict flow cytometry analysis of cell surface markers on cells taken from a day 9 hemangioblast enriched cultures.

[0049] FIGS. 3A-I depict flow cytometry analysis of cell surface markers on cells taken from day 14 hemangioblast enriched cultures.

[0050] FIG. 4 shows the karyotyping of passage-10 hES-MS-C derived from the H9-hESC line.

[0051] FIGS. 5A-C shows the disease scores of EAE mice treated with hES-MS-Cs prior to the onset of clinical disease. 10⁶hES-MS-C or undifferentiated hESC or saline control (PBS) was i.p. injected into the mice 6 days after the EAE inducing immunization. Panel A shows mice injected with hES-MS-Cs (CT2), panel B shows mice injected with hES-MS-Cs (MA09), and panel C shows mice injected with hES-MS-Cs (H9). N=5 mice per group, ***P<0.001.

[0052] FIGS. 6A-F are bar graphs depicting cumulative disease score (panels A-C) and the maximal disease scores (panels D-F) from days 28-32 post immunization for the mice shown in FIG. 5. N=5 mice per group, **P<0.01.

[0053] FIG. 7 is a graph of disease scores of EAE mice treated with hES-MS-C or saline control (PBS) post-clinical disease onset. 10⁶hES-MS-C were i.p injected into mice 18 days post-immunization. N=6 mice per group, ***P<0.001.

[0054] FIGS. 8A-B show flow cytometric analyses of regulatory T cells (CD25⁺FoxP3⁺) in the CNS of EAE mice treated with saline (PBS) or hES-MS-Cs derived from hESC line CT2 15 days after immunization.

[0055] FIGS. 9-F show a bar graph depicting the total numbers of CD4⁺, CD8⁺ cells, Th1 CD4⁺ T cells, and Th17 CD4⁺ T cells in the CNS of EAE mice treated with saline control (PBS), hESC or hES-MS-C on day 15 post-immunization (panels A-D). Panels E-F show the expression of IL-17 and IFN-gamma in CD4⁺ T cells from PBS or hES-MS-C treated EAE mice. N=4 mice per group, *P<0.05, **P<0.01.

[0056] FIGS. 10A-D show immunohistochemical detection of myelin basic protein (MBP; red), CD3 for T cells (green) and IBA1 for microglia (green) on lumbar spinal cord cross sections from EAE mice treated with either hES-MS-C (panels a and c) or saline (PBS) (panels b and d).

[0057] FIG. 11 shows a quantitative analysis of myelin basic protein (MBP) in the spinal cord was performed using relative fluorescent intensity (RFI) measurement of MBP

US 2015/0203820 A1

Jul. 23, 2015

5

expression in digitally captured spinal cord hemisections. N=4 to 6 mice per group, **P<0.02.

[0058] FIGS. 12A-C show graphs of disease scores of EAE mice treated with saline (PBS), bone marrow derived MSCs (BM-MSC) or hES-MSC prior to the onset of clinical disease. Panel A shows 5 groups of mice treated with either PBS, hES-MSCs (MA09) or BM-MSCs from one of three different sources. Panel B shows mice treated with PBS, BM-MSC or hES-MSC (CT2) prior to clinical disease onset. Panel C shows mice treated with PBS, BM-MSC or hES-MSC (MA09). For all experiments shown, N=5 mice per group, ***P<0.001 between hES-MSC and any of the three BM-MSC treated groups.

[0059] FIGS. 13A-D shows the total number of CD4⁺, CD8⁺, Th1 or Th7 T cells in the CNS of EAE mice treated with saline control (PBS), BM-MSCs (BM-MSC lines 1, 2 or 3) or hES-MSC. (N=4 mice per group, *P<0.05, **P<0.01).

[0060] FIGS. 14A-E show the qualitative analysis of myelin content in spinal cord cross-sections of EAE mice treated with saline (PBS), BM-MSCs (BM-MSC lines 1, 2, or 3), or hES-MSCs using Fluoromyelin stain (green) and counterstained with DAPI (blue) to indicate infiltration of nucleated cells.

[0061] FIGS. 15A-B show the localization of fluorescently labeled hES-MSC or BM-MSC in spinal cord cryosections (60 μ m) taken from EAE mice 14 post immunization. Mice received an i.p. administration of GFP⁺hES-MSC or GFP⁺BM-MSC or PBS control. Mice were euthanized following the MSC cell administration and immunostained for GFP (green; to track the injected hES-MSC or BM-MSC cells), CD31 (red; vascular) and DRAQ5 (blue; cell nuclei). Panel A is parenchymal inflamed venules. Panel B shows meningeal venules. Isosurface rendered 3D reconstruction of the selected ROI (white dotted box) are shown next to the original images for enhanced spatial perspective. The insets show the GFP-DRAQ5 (upper inset image) and isolated GFP (lower inset image) channels separately.

[0062] FIGS. 16A-B show the proportion of proliferating CD4⁺ or CD8⁺ T cells, respectively, co-cultured in vitro with one of two hES-MSCs (MA09 or CT2) or one of three BM-MSC lines (1, 2 or 3) or no MSCs (PBS). T cells were stimulated with the indicated concentration of anti-CD3 antibody and proliferation was measured by CFSE dilution using flow cytometry. T cells and MSCs were mixed at a ratio of 10:1. N=3 replicates per group.

[0063] FIG. 17 shows the proliferation of CD4⁺ or CD8⁺ T cells co-cultured with BM-MSC, hES-MSC or no MSC (control) and stimulated with 0 μ g/ml (NC), 0.1 μ g/ml or 0.3 μ g/ml anti-CD3 antibody. Flow cytometry histogram plots show the percentage of divided CD4⁺ or CD8⁺ T cells with diluted CFSE signal.

[0064] FIGS. 18A-J depicts intracellular FACS staining of IFN γ ⁺ or IL-17⁺ naive CD4⁺ T cells co-cultured with hES-MSC or one of three BM-MSC cell lines (#2, #3, or #6) or no MSCs (control) and stimulated with TPA/ionomycin stimulation of hES- or BM-MSC incubated with mouse naïve CD4⁺ T cells, followed by Th1 or Th17 differentiation for 5 days. Data shown are from 1 of 4 independent experiments.

[0065] FIGS. 19A-B show relative gene expression levels from hES-MSC or BM-MSC as determined by microarray analysis. N=2, *P<0.05, **P<0.01.

[0066] FIGS. 20A-F show the expression of IL-6 and IL-10 in 3 individual BM-MSC and 3 individual hES-MSC lines by intracellular FACS staining.

[0067] FIGS. 21A-D shows the expression of IL-6 by intracellular FACS staining of IL-6 in BM-MSC or hES-MSC (CT2) cultured with IFN γ . NC is negative control.

[0068] FIGS. 22A-C show the percent of proliferating CFSE labeled CD8⁺ T cells stimulated with various doses of anti-CD3 antibody and co-cultured with or without one of three BM-MSC lines (#2, #3 or #6) at a ratio of 10:1. Anti-human IL-6 antibody (10 μ g/ml) or isotype control (IgGk) was added to the cultures as indicated. N=4 replicates per data point, **P<0.01.

[0069] FIGS. 23A-B show that IL-6 neutralizing antibody (α IL-6) enhances suppression of BM-MSC on CD4 and CD8 T cell proliferation in vitro; NC=T cells cultured without MB-MSC or anti-IL6.

[0070] FIGS. 24A-J show the proportion of IFN γ ⁺ or IL-17⁺ CD4⁺ T cells detected via intracellular FACS staining after TPA/ionomycin stimulation in vitro. hES- or BM-MSC were incubated with mouse naïve CD4⁺ T cells at a ratio of 1:10 under the Th17 differentiation conditions for 5 days, in the presence or absence of 10 μ g/ml anti-human IL-6 antibody.

[0071] FIG. 25 shows the clinical disease scores of EAE mice injected with irradiated hES-MSC (Irr-hES-MSC; from MA09), hES-MSCs (from MA09) or saline (PBS). N=5 mice per group, ***P<0.001.

[0072] FIG. 26 shows immunostaining of luciferase-expressing hES-MSC (CT2). The luciferase expressing hES-MSCs cultured in Petri dish were immunostained with an anti-luciferase antibody (green) and counterstained for nuclei with DAPI (blue).

[0073] FIGS. 27A-B show the localization of non-irradiated hES-MSC or irradiated (Irr-hES-MSC) expressing D-Luciferin at various days following injection into EAE mice. Images were taken using the Xenogen IVIS 100 system. Non-irradiated (panels A) and irradiated (panels B) luciferase-expressing hES-MSCs (CT2) are shown in the dorsal and ventral images of EAE mice.

[0074] FIGS. 28A-D show the effect of the GSK3 inhibitor BIO ((2'Z,3'E)-6-Bromoindirubin-3'-oxime, 6-BIO) on the differentiation of embryoid bodies (EB) from hES cells. BIO significantly increases BM formation and the number of cells obtained by day 7 in culture

[0075] FIG. 29 shows a bar chart show BIO increase the EB formation numbers

[0076] FIG. 30 shows flow cytometry plots showing BIO treatment increases the hemangioblast forming efficiency.

[0077] FIG. 31 shows microarray analysis of CD10 expression level of different hES-MSC lines and BM-MSC lines.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1 Definitions

[0078] The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the methods of the invention and how to use them. Moreover, it will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed

US 2015/0203820 A1

Jul. 23, 2015

6

herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of the other synonyms. The use of examples anywhere in the specification, including examples of any terms discussed herein, is illustrative only, and in no way limits the scope and meaning of the invention or any exemplified term. Likewise, the invention is not limited to its preferred embodiments.

[0079] The term hESC means human embryonic stem cells that encompass pluripotent stem cells produced from embryo, inner cell mass, blastomere or a cell line.

[0080] The term “hES-HB-MS” are mesenchymal stem cells that are derived via hemangioblast or hemangio-colony forming middle step.

[0081] The term “hES-MS” or hES-MSs” or “human embryonic mesenchymal stem cells” or human embryonic stem cell derived mesenchymal stem cells” or “hES-MS population” as used herein means mesenchymal-like stem cells, mesenchymal-like stromal cells, mesenchymal stem cells or mesenchymal stromal cells, derived from human embryonic stem cells or derived from induced pluripotent stem cells using any methods. hES-MS as used herein includes individual cells, cell lines, batches, lots or populations of hES-MS.

[0082] The term “clinical grade hES-MS” as used herein means hES-MS which contains characteristics that are suitable for use in clinical use for human, avian or other mammals. Clinical grade hES-MS as used herein includes individual cells, cell lines, batches, lots or populations of MS.

[0083] The term “hES-MS population” as used herein means a population of hES-MS cells which contains cells that have characteristics that are suitable for use in treatment and cells that do not have characteristics that are suitable for use in treatment.

[0084] The terms “iPS-MS” and “iPS-MSs” and “human induced pluripotent stem cells derived mesenchymal stem cells” are to be used interchangeably throughout. These cells can be described based upon numerous structural and functional properties including but not limited to, expression or lack of expression of one or more markers. iPS-MSs are multipotent and capable of differentiating to give rise to cell types of other lineages.

[0085] The phrase “therapeutically effective amount” is used herein to mean an amount sufficient to cause an improvement in a clinically significant condition in the subject, or delays or minimizes or mitigates one or more symptoms associated with the disease, or results in a desired beneficial change of physiology in the subject.

[0086] The terms “treat”, “treatment”, and the like refer to a means to slow down, relieve, ameliorate or alleviate at least one of the symptoms of the disease, or reverse the disease after its onset.

[0087] The terms “prevent”, “prevention”, and the like refer to acting prior to overt disease onset, to prevent the disease from developing or minimize the extent of the disease or slow its course of development.

[0088] The term “subject” as used in this application means an animal with an immune system such as avians and mammals. Mammals include canines, felines, rodents, bovine, equines, porcines, ovines, and primates. Avians include, but are not limited to, fowls, songbirds, and raptors. Thus, the invention can be used in veterinary medicine, e.g., to treat companion animals, farm animals, laboratory animals in zoological parks, and animals in the wild. The invention is particularly desirable for human medical applications

[0089] The term “in need thereof” would be a subject known or suspected of having or being at risk of developing a disease including but not limited to multiple sclerosis and other T cell related autoimmune diseases, or diseases related to the central nervous system or the blood-brain barrier or the blood-spinal cord barrier.

[0090] A subject in need of treatment would be one that has already developed the disease. A subject in need of prevention would be one with risk factors of the disease.

[0091] The term “agent” as used herein means a substance that produces or is capable of producing an effect and would include, but is not limited to, chemicals, pharmaceuticals, drugs, biologics, small molecules, antibodies, nucleic acids, peptides, and proteins.

5.2 In Vitro Differentiation of Embryonic Stem Cells Through Hemangioblasts to Obtain Mesenchymal-Like Stem Cells

[0092] The present invention provides a method for generating and expanding mesenchymal-like stem cells (MSCs) from human hemangioblasts (HB) derived from embryonic stem cells (hES). These resulting cells are designated hES-MSs. These hES-MSs can be isolated and/or purified.

[0093] MSC-like cells have been derived from human embryonic stem cells by various methods (Barbieri et al. (2005); Olivier et al. (2006); Sanchez et al. (2011); Brown et al. (2009)). However, all of these methods involve co-culturing and hand-picking procedures that limit yield and purity and result in varying quality of cells.

[0094] To solve these problems, the method of the current invention derives MSCs from embryoid bodies and then hemangioblasts. It was hypothesized that because mesenchymal cells and hemangioblasts both originate from the mesodermal progenitors (MP) (Huber et al. (2004)), that a method to derive HB from hESC via EB would actually enrich MP that can further differentiate into either HB or MSCs depending on subsequent culture conditions. As shown in FIG. 1, MSCs were obtained by using a hESC to EB to HB to MSCs.

[0095] Although hESC express low levels of MHC antigens, it has been found that many cell types differentiated from hESC have increased expression of these antigens (Draper et al., 2002; Drukker et al., 2006; Drukker et al., 2002), thus, causing great concern for immunorejection of the differentiated cells if transplanted into patients. In contrast, MSC express low levels of costimulatory molecules and major MHC antigens, and have been used in allogeneic or xenograft models to treat autoimmune diseases (Gordon et al., 2008b; Grinnemo et al., 2004; Rafei et al., 2009a; Rafei et al., 2009b; Tse et al., 2003). hES-MSs, like adult tissue-derived MSC, express low levels of the co-stimulatory molecules and MHC antigens, and do not require long-term engraftment to exert immunosuppressive effect, thus, there is no concern for immunorejection due to mismatch of MHC antigens between MSC and the recipient (Ohtaki et al., 2008; Uccelli and Prockop, 2010a). One hESC line is sufficient to generate hES-MS at large scale, in an endless supply, and with easy quality control, suitable for industrial production as a potential therapy to treat patients with MS and other T cell-based autoimmune diseases.

[0096] The methods for obtaining EB from hES and then dissociating the EB into HB has been previously reported in Lu et al. (2007) and Lu et al. (2008) as well as in United States Patent Application Publication No. 2012/0027731 all hereby incorporated herein in their entirety.

US 2015/0203820 A1

Jul. 23, 2015

7

[0097] Human hemangio-colony forming cells can be generated from human embryonic stem cells. Such embryonic stem cells include embryonic stem cells derived from or using, for example, blastocysts, plated ICMs, one or more blastomeres, or other portions of a pre-implantation-stage embryo or embryo-like structure, regardless of whether produced by fertilization, somatic cell nuclear transfer (SCNT), parthenogenesis, androgenesis, or other sexual or asexual means.

[0098] Additionally or alternatively, hemangio-colony forming cells can be generated from other embryo-derived cells. For example, hemangio-colony forming cells can be generated (without necessarily going through a step of embryonic stem cell derivation) from or using plated embryos, ICMs, blastocysts, trophoblast/trophectoderm cells, one or more blastomeres, trophoblast stem cells, embryonic germ cells, or other portions of a pre-implantation-stage embryo or embryo-like structure, regardless of whether produced by fertilization, somatic cell nuclear transfer (SCNT), parthenogenesis, androgenesis, or other sexual or asexual means. Similarly, hemangio-colony forming cells can be generated using cells or cell lines partially differentiated from embryo-derived cells. For example, if a human embryonic stem cell line is used to produce cells that are more developmentally primitive than hemangio-colony forming cells, in terms of development potential and plasticity, such embryo-derived cells could then be used to generate hemangio-colony forming cells.

[0099] Additionally or alternatively, hemangio-colony forming cells can be generated from other pre-natal or perinatal sources including, without limitation, umbilical cord, umbilical cord blood, amniotic fluid, amniotic stem cells, and placenta.

[0100] It is noted that when hemangio-colony forming cells are generated from human embryonic tissue a step of embryoid body formation may be needed. However, given that embryoid body formation serves, at least in part, to help recapitulate the three dimensional interaction of the germ layers that occurs during early development, such a step is not necessarily required when the embryo-derived cells already have a structure or organization that serves substantially the same purpose as embryoid body formation. By way of example, when hemangio-colony forming cells are generated from plated blastocysts, a level of three dimensional organization already exists amongst the cells in the blastocyst. As such, a step of embryoid body formation is not necessarily required to provide intercellular signals, inductive cues, or three dimensional architecture.

[0101] Hemangio-colony forming cells can be generated from embryo-derived cells. In certain embodiments, the embryo-derived cells are embryonic stem cells. In certain other embodiments, the embryo-derived cells are plated embryos, ICMs, blastocysts, trophoblast/trophectoderm cells, one or more blastomeres, trophoblast stem cells, or other portions of an early pre-implantation embryo. For any of the foregoing, the embryo-derived cells may be from embryos produced by fertilization, somatic cell nuclear transfer (SCNT), parthenogenesis, androgenesis, or other sexual or asexual means.

[0102] The human embryonic stem cells or induced pluripotent stem cell may be the starting material of this method. The embryonic stem cells or iPS may be cultured in any way known in the art, such as in the presence or absence of feeder cells. Adding GSK3 inhibitor BIO at 0.05 uM-0.2 uM can

increase the embryoid body formation and subsequent hemangioblast forming efficiency, shortening the culture time.

[0103] In the examples set forth herein, four hESC cell lines were used, H9 (derived from WiCell Research Institute) (Thomson et al. (1998), CT2 (derived from University of Connecticut Stem Cell Core (Lin et al. (2010)); MA09 (an FDA approved, clinical-grade cell line derived at Advanced Cell Technology, Inc.) (Klimanskaya et al (2006)); and ES03-Envy (Envy, a GFP-labeled line, derived at ES International) (Costa et al. (2005)).

[0104] In the first step of this method for generating and expanding human hemangioblast cells to obtain MSCs, human stem cells are grown in serum-free media and are induced to differentiate into embryoid bodies. To induce embryoid body formation, embryonic stem cells may be pelleted and resuspended in serum-free medium such as DMEM/F12, HPGM(Lonza), StemSpan H3000 (Stemcell Technologies), Stempro-34, QBSF-60, Xvivo-15, IMDM, Stemline I or II media (Sigma™) supplemented with one or more morphogenic factors and cytokines and then plated on low attachment culture dishes. Morphogenic factors and cytokines may include, but are not limited to, bone morphogenic proteins (e.g., BMP-2, BMP-4, or BMP-7, but not BMP-3) and VEGF, SCF and FL. Bone morphogenic proteins and VEGF may be used alone or in combination with other factors. The morphogenic factors and cytokines may be added to the media from 0-72 hours of cell culture. Following incubation under these conditions, incubation in the presence of early hematopoietic expansion cytokines, including, but not limited to, thrombopoietin (TPO), Flt-3 ligand, and stem cell factor (SCF), allows the plated ES cells to form EBs. In addition to TPO, Flt-3 ligand, and SCF, VEGF, BMP-4, may also be added to the media. In one embodiment, human ES cells are first grown in the presence of BMP-4 and VEGF₁₆₅ (e.g., 25-100 ng/ml), followed by growing in the presence of BMP-4, VEGF₁₆₅, SCF, TPO, and FLT3 ligand (e.g., 10-50 ng/ml). The additional factors may be added 48-72 hours after plating.

[0105] Next, human hemangioblast cells are isolated from early embryoid bodies (EBs). Isolating hemangioblast cells from early EBs supports the expansion of the cells in vitro. For human cells, hemangioblast cells may be obtained from EBs grown for less than 10 days. In certain embodiments of the present invention, hemangioblast cells arise in human EBs grown for 2-6 days. According to one embodiment, hemangioblast cells are identified and may be isolated from human EBs grown for 4-6 days. In other embodiments, human EBs are grown for 2-5 days before hemangioblast cells are isolated. In certain embodiments, human EBs are grown for 3-4.5 days before hemangioblast cells are isolated.

[0106] In an embodiment of the method, early EBs are washed and dissociated, with TrypLE-LE (Invitrogen), Trypsin/EDTA or collagenase B. A select number of cells (e.g., 2.5×10^5 cells) are then mixed with serum-free methylcellulose medium optimized for hemangioblast cell growth, such as BL-CFU medium, for example Stem Cell Technologies Catalogue H4436, H4536, or hemangioblast cell expansion medium (HGM), or any medium containing 1.0% methylcellulose in MDM, 1-2% Bovine serum albumin, 0.1 mM 2-mercaptoethanol, 10 µg/ml rh-Insulin, 200 µg/ml iron saturated human transferrin, 20 ng/ml rh-GM-CSF, 20 ng/ml rh-IL-3, 20 ng/ml rh-IL-6, 20 ng/ml rh-G-CSF (“rh” stands for “recombinant human”). This medium may be supplemented with early stage cytokines including, but not limited

US 2015/0203820 A1

Jul. 23, 2015

8

to, EPO, TPO, SCF, FL, Flt-3, VEGF, BMPs such as BMP2, BMP4 and BMP7, but not BMP3 and HOXB4 (or another homeobox protein). In certain embodiments, erythropoietin (EPO) is added to the media. In further embodiments, EPO, SCF, VEGF, BMP-4 and HoxB4 are added to the media. In additional embodiments, the cells are grown in the presence of EPO, TPO and FL. In certain embodiments where H9 is the starting human ES cell line, EPO, TPO and FL are added to the media. In addition to EPO, TPO and FL, media for cells derived from H9 or other ES cells may further comprise VEGF, BMP-4, and HoxB4.

[0107] The cells so obtained by this method (the cells may be in BL-CFU medium), which include hemangioblast cells, are plated onto ultra-low attachment culture dishes and incubated in a CO₂ incubator to grow hemangioblast colonies. Some cells may be able to form secondary EBs. Following approximately 3-6 days, and in some instances 3-4.5 days, hemangioblast colonies are observed. Hemangioblast colonies may be distinguished from other cells such as secondary EBs by their distinctive grape-like morphology and/or by their small size. In addition, hemangioblasts may be identified by the expression of certain markers (e.g., the expression of both early hematopoietic and endothelial cell markers) as well as their ability to differentiate into at least both hematopoietic and endothelial cells. For example, while hemangioblasts lack certain features characteristic of mature endothelial or hematopoietic cells, these cells may be identified by the presence of certain markers (such as, for example, CD133, SCA-1, CD34, CD45, CD31, cKit, Nestin⁺, Stro-1, Stro-3, CD71⁺). Hemangioblasts may also express GATA-1 and GATA-2 proteins, CXCR-4, and TPO and EPO receptors. Further, hemangioblasts may be characterized by the expression of certain genes, those genes associated with hemangioblasts and early primitive erythroblast development, such as, for example, SCL, LMO2, FLT-1, embryonic fetal globin genes, NF-E2, GATA-1, EKLF, ICAM-4, glycophorins, and EPO receptor).

[0108] The hemangioblast cells may be isolated by size and/or morphology by the following procedure. After 3 to 7 days of growth, the cell mixture contains EBs, which are round and represent a clump of multiple cells, and hemangioblasts, which are grape-like, smaller than the EBs, and are single cells. Accordingly, hemangioblasts may be isolated based on their morphology and size. The hemangioblast cells may be manually picked, for example, when observing the cell mixture under a microscope. The cells may subsequently grow into colonies, each colony having between 100-150 cells.

[0109] In one embodiment, hemangioblast cells are digested to form single cells with TrypLE, Trypsin or collagenase B. The single cells are re-suspended in a medium optimized for mesenchymal stem cell growth such as alpha-MEM containing 2-20% of fetal bovine serum, human AB serum, DMEM-high glucose containing 2-20% of fetal bovine serum, the FBS can be replaced with 5-20% of knock-out serum replacement (KOSR) or bovine serum albumin (BSA), or any other commercial available serum free MSC culture mediums. In certain embodiments, Serum, KOSR or BSA is added in a concentration of from about 5-20%. In certain embodiments, Fetal bovine serum is preferred. In certain embodiments, cells are cultured at a density of about 10-1000 cells/cm². In certain embodiments, the cells are cultured in an environment that mimics the extracellular environment of tissues, such as Matrigel.

[0110] After approximately 24 hours, a number of cells (5-10%) attached to the culture plate and approximately 6-14 days later, mesenchymal stem cells begin to differentiate from the HBs. MSCs are identified by a small cell with spindle-like morphology. MSCs can also be identified by the cell surface expression of CD146 and CD166 and by the absence or low expression of certain cell surface markers such as CD31, CD34, and CD45. MSCs are also characterized as multipotent and able to differentiate into chondrocytes, osteocytes, and adipocytes. CD10 expression level may be different from the cell source, CT2 and H9-MSC express low level of CD10 whereas MA09-MSC express high level of CD10, which is not related to its immunosuppressive function but may related to other function like fibrosis and downstream differentiation. In certain embodiments, less than 28%, 27%, 26%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1% of the hES-MSC express CD10.

[0111] In a further embodiment of the present invention, an additional step of irradiating the hES-MSCs is performed. This irradiation can be accomplished with the use of any method known in the art that emits radiation including but not limited to gamma irradiation e.g. Cesium-137 gamma irradiation, or photon radiation using X-ray. The preferred amount of radiation to be administered is about between 5 and 20000 Gy, more preferably about between 50 and 100 Gy, and most preferably 80 Gy.

5.3 Human Embryonic Stem Cell Derived Mesenchymal Stem Cells (hES-MSC) and iPS-MSC

[0112] One embodiment of the present invention is the human embryonic stem cell derived mesenchymal stem cells, designated hES-MSC. These cells are unique and have a variety of therapeutic and other uses. Thus, the present invention included various preparations, including pharmaceutical preparations, and compositions comprising hES-MSCs.

[0113] The terms “hES-MSC” and “hES-MSCs” and “human embryonic stem cell derived mesenchymal stem cells” and “human embryonic mesenchymal stem cells” will be used interchangeably throughout. These cells can be described based upon numerous structural and functional properties including but not limited to, expression or lack of expression of one or more markers. hES-MSCs are multipotent and capable of differentiating to give rise to cell types of other lineages.

[0114] The terms “iPS-MSC” and “iPS-MSCs” and “human induced pluripotent stem cells derived mesenchymal stem cells” will be used interchangeably throughout. These cells can be described based upon numerous structural and functional properties including but not limited to, expression or lack of expression of one or more markers. iPS-MSCs are multipotent and capable of differentiating to give rise to cell types of other lineages.

[0115] Human embryonic stem cell derived mesenchymal stem cells are identified and characterized based upon their structural properties. Specifically, hES-MSCs are characterized by small cell bodies with a fibroblast like and spindle like morphology.

[0116] hES-MSCs and iPS-MSC are identified or characterized by the expression or lack of expression as assessed on the level of DNA, RNA or protein, of one or more cell markers. hES-MSCs can be identified as expressing cell surface marker CD73, or expressing at least one or more of the following cell surface markers: CD90, CD105, CD13, CD29, CD54, CD44, or CD146 and CD166 or not expressing or

US 2015/0203820 A1

Jul. 23, 2015

9

expressing at a low level at least one of the following cell surface markers: CD34, CD31, or CD45.

[0117] Alternatively or additionally, hES-MSCs are identified or characterized based upon their low level of expression of one or more pro-inflammatory proteins, MMP2, RAGE, IFN- γ R1, IFN γ R2, IL-12, TNF α , IL-6, and VCAM1. This profile of gene expression is in contrast to bone marrow derived mesenchymal stem cells. In particular, IL-6 was expressed much higher in BM-MSCs than in hES-MSCs. IL-6 is a pleiotropic cytokine involved in crosstalk between hematopoietic/immune cells and stromal cells, including the onset and resolution of inflammation.

[0118] Alternatively or additionally, hES-MSCs, similar to other kind of MSC, are identified by the expression of high levels of immunosuppressive non-classical MHC antigen HLA-G and HLA-ABC and low levels or no expression of MHC class-II antigen HLA-DR and co-stimulatory molecule CD80.

[0119] The hES-MSCs are also characterized in their ability to inhibit T cell proliferation after stimulation in vitro. This characteristic is in contrast to BM-MSCs which has less potency in inhibiting T cell proliferation after stimulation in vitro.

[0120] Thus, the human embryonic stem cell-derived mesenchymal stem cells described herein have at least one of the following characteristics: 1. Differentiate into chondrocytes, osteocytes and/or adipocytes; 2. Have a fibroblast-like morphology; 3. Express CD73, CD90, CD105, CD13, CD29, CD54, CD44, CD146 and/or CD166; 5. Express at low levels or do not express CD34, CD31, and/or CD45; 6. Express at low levels or do not express MMP2, RAGE, IFN γ R1, IFN γ R2, IL-12, TNF α , IL-6, and/or VCAM1, particularly IL-6; 7. Express MHC antigen HLA-G and/or HLA-ABC and express at low levels or do not express HLA-DR and/or CD80; and 8. Inhibit T cell proliferation after stimulation in vitro. In certain embodiments, the hES-MSCs have at least two, at least three, at least four, at least five, at least six, at least seven or all eight six characteristics.

[0121] Additionally, the human embryonic stem cell derived mesenchymal stem cells described herein have the unique ability to cross the blood-brain barrier (BBB) and the blood-spinal cord barrier (BSCB), making them uniquely suited for therapeutic and diagnostic applications. As shown herein, the hES-MSCs described herein have the ability to migrate in and out of the vessels of the spinal cord, across the BSCB, to fulfill functions in the CNS, including but not limited to the delivery of therapeutic and diagnostic agents. This is in contrast to BM-MSCs which do not have this ability.

[0122] In certain embodiments, the hES-MSC is irradiated either by gamma or x-ray radiation. This embodiment would include human embryonic stem cell derived mesenchymal stem cells with at least one of the following characteristics listed above, having at least two, at least three, at least four, at least five, at least six, at least seven, all eight characteristics and that the hES-MSC have been subjected to irradiation.

[0123] In another embodiment, the cell culture comprises human embryonic stem cell derived mesenchymal stem cells. In certain embodiments, the hES-MSCs differentiate into chondrocytes, osteocytes and/or adipocytes. In certain embodiments, the cells express CD73, CD90, CD105, CD13, CD29, CD54, CD44, CD146, and/or CD166. In certain embodiments, the cells express at low levels or do not express CD34, CD31, and/or CD45. In certain other embodiments, the cells express at low levels or do not express MMP2,

RAGE, IFN γ R1, IFN γ R2, IL-12, TNF α , IL-6, and/or VCAM1, especially IL-6. In certain other embodiments, the cells express MHC antigen HLA-G and/or HLA-ABC and express at low levels or do not express HLA-DR and/or CD80. In certain other embodiments, the cells inhibit T cell proliferation after stimulation in vitro. In certain embodiments, the cells can cross the blood-brain barrier and the blood-spinal cord barrier. In certain embodiments, the cells have been irradiated.

[0124] In another aspect, provided herein is a pharmaceutical preparation comprising human embryonic stem cell derived mesenchymal stem cells. In certain embodiments, the hES-MSCs can differentiate into chondrocytes, osteocytes and/or adipocytes. In certain embodiments, the cells express CD73, CD90, CD105, CD13, CD29, CD54, CD44, CD146 and/or CD166. In certain embodiments, the cells express at low levels or do not express CD34, CD31, and/or CD45. In certain other embodiments, the cells express at low levels or do not express MMP2, RAGE, IFN γ R1, IFN γ R2, IL-12, TNF α , IL-6, and/or VCAM1, especially IL-6. In certain other embodiments, the cells express MHC antigen HLA-G and/or HLA-ABC and express at low levels or do not express HLA-DR and/or CD80. In certain other embodiments, the cells inhibit T cell proliferation after stimulation in vitro. In certain embodiments, the cells can cross the blood-brain barrier and the blood-spinal cord barrier. In certain embodiments, the cells have been irradiated. The pharmaceutical preparation can be prepared using any pharmaceutically acceptable carrier or excipient.

[0125] In certain embodiments, the composition or pharmaceutical preparation comprises at least at least 10,000 human embryonic-mesenchymal stem cells, at least 50,000 human embryonic-mesenchymal stem cells, at least 100,000 human embryonic-mesenchymal stem cells, at least 500,000 human embryonic-mesenchymal stem cells, at least 1×10^6 human embryonic-mesenchymal stem cells, at least 5×10^6 human embryonic-mesenchymal stem cells, at least 1×10^7 human embryonic-mesenchymal stem cells, at least 5×10^7 human embryonic-mesenchymal stem cells, at least 1×10^8 human embryonic-mesenchymal stem cells, at least 5×10^8 human embryonic-mesenchymal stem cells, at least 1×10^9 human embryonic-mesenchymal stem cells, at least 5×10^9 human embryonic-mesenchymal stem cells, or at least 1×10^{10} human embryonic-mesenchymal stem cells.

[0126] In certain embodiments, the invention provides a cryopreserved preparation of human hemangio-colony cells or cells partially or terminally differentiated therefrom.

[0127] In certain embodiments, the invention provides the therapeutic use of hES-MSCs, or compositions or preparations of hES-MSCs, including irradiated hES-MSCs. Such cells and preparations can be used in the treatment of any of the conditions or diseases detailed throughout the specification, as well as in a delivery system for agents across the blood-brain barrier and the blood-spinal cord barrier.

5.4 Selecting and Producing hES-MSC Populations

[0128] Provided herein is a method of identifying highly immunosuppressive hES-MSC by identifying a biomarker profile of the highly immunosuppressive hES-MSC that are clinical grade for use in therapy. In certain embodiment, the clinical grade hES-MSC have the following characteristics: (i) contain >95% of cells expressing group-1 markers; (ii) contain >80% of cells expressing group 2 markers; (iii) contain <5% of cells expressing group-3 markers (iv) expressing

US 2015/0203820 A1

Jul. 23, 2015

10

IL-10 and TGF β ; (v) contain <2% of cells expressing IL-6, IL-12 and TNF α ; and (vi) contains <0.001% of cells co-expressing all group-4 markers, wherein group-1 markers are CD73, CD90, CD105, CD146, CD166, and CD44, group-2 markers are CD13, CD29, CD54, CD49E, group-3 markers are CD45, CD34, CD31 and SSEA4, and group-4 markers are OCT4, NANOG, TRA-1-60 and SSEA4.

[0129] In certain embodiments, the method comprises measuring the differential expression of markers that encode anti-inflammatory factors ("AIF") and pro-inflammatory factors ("PIF"). In certain embodiments, the AIF is IL-10, TGF β 2. In certain embodiments, the PIF is upregulated. In certain embodiments, hES-MSC express at least 1.5 fold of the above markers as compared to BM-MSC. In certain embodiments, the PIF is IL-6, IL-12, TNF α , CCL2, VCAM1, RAGE, MMP2. In certain embodiment, the PIF is downregulated. In certain embodiments, hES-MSC express at least half of the above markers as compared to BM-MSC. In another embodiment, highly immunosuppressive hES-MSC has a lower ratio of IL-6⁺ cells as compared to BM-MSC. In certain embodiments, highly immunosuppressive hES-MSC has less than 5%, 4%, 3%, 2%, 1% of IL-6 positive cells. In certain embodiment, hES-MSC express low level of IL12, TNF α , RAGE and other PIF. In certain embodiment, hES-MSC may express high level of TGF β 2 and IL-10. In certain embodiments, the expression of markers are compared to expression in BM-MSC.

[0130] Provided herein is a qualification procedure for clinical grade hES-MSC population. Expression of specific markers are measured in a population of hES-MSC to determine whether they are suitable for therapeutic use. The markers include, for example, (1) MSC-specific markers (set 1): CD73, CD90, CD105, CD166, and CD44, (2) MSC-specific markers (set 2): CD13, CD29, CD54, CD49E, SCA-1, and STRO-1, (3) hematopoietic stem/progenitor markers: CD45 and CD34, and endothelial cell marker CD31, (4) immunogenic markers: HLA-ABC, HLA-G, CD80, and CD86, (5) cytokines: IL-10, TGF β , IL-6, and IL-12, and (6) pluripotency markers: OCT4, NANOG, TRA-1-60, and SSEA-4. In certain embodiments, hES-MSC population contains more than 95%, 96%, 97%, 98%, 99% of cells that express at least one group 1 markers. In certain embodiments, hES-MSC population contains more than 80%, 85%, 90%, 95%, 99% of cells that express at least one group 2 markers. In certain embodiments, hES-MSC population contains less than 0.1%, 0.08%, 0.05%, 0.03%, 0.02%, 0.01% of cells that express at least one group 3 marker. In certain embodiments, hES-MSC population contains more than 80%, 85%, 90%, 95%, 99% of cells that express IL-10 and/or TGF β . In certain embodiments, hES-MSC population contains less than 5%, 4%, 3%, 2%, 1% of cells that express IL-6 and/or IL-12. In certain embodiments, hES-MSC population contains less 0.001% of cells that express at least one group 6 marker. The clinical-grade hES-MSC is compared with the preclinical-grade hES-MSC as a positive control. In certain embodiment, the hES-MSC is characterized through multi-color flow cytometry analyses and/or immunofluorescence. In certain embodiments, hES-MSC population express CCL2, CCL3, CCL4, CCL5, IL-1, IL-2, IL-4, IL-6, IL-8, IL-17, TNF α , TGF β , IFN γ , GM-CSF, G-CSF, bFGF, CXCL5, VEGF, TPO or a combination thereof. In certain embodiments, the hES-MSC population will also be analyzed for (1) presence of exogenous materials such as endotoxin and residual cytok-

ines/growth factors, and/or (2) genomic abnormalities (via karyotyping and whole-genome sequencing).

[0131] Methods for determining the expression profile of the MSC are known in the art, including but not limited to, flow cytometry, multiplex microarray, RT-PCT, northern blot and western blot. In certain embodiments, the expression profile of the MSC are determined by cytometric bead array based multiplex cytokine analysis, luminex system based multiplex cytokine analysis, microarray RNA-seq, quantitative RT-PCR, Elispot Elisa, Elisa cytokine array, flow cytometry luciferase reporter system, fluorescence reporter system, histology staining, and Immunofluorescence staining.

5.4.1 Methods of Detecting Nucleic Acid Biomarkers

[0132] In specific embodiments, biomarkers in a biomarker profile are nucleic acids. Such biomarkers and corresponding features of the biomarker profile may be generated, for example, by detecting the expression product (e.g., a polynucleotide or polypeptide) of one or more markers. In a specific embodiment, the biomarkers and corresponding features in a biomarker profile are obtained by detecting and/or analyzing one or more nucleic acids expressed from a marker disclosed herein using any method well known to those skilled in the art including, but not limited to, hybridization, microarray analysis, RT-PCR, nuclease protection assays and Northern blot analysis.

[0133] In certain embodiments, nucleic acids detected and/or analyzed by the methods and compositions of the invention include RNA molecules such as, for example, expressed RNA molecules which include messenger RNA (mRNA) molecules, mRNA spliced variants as well as regulatory RNA, cRNA molecules (e.g., RNA molecules prepared from cDNA molecules that are transcribed in vitro) and discriminating fragments thereof.

[0134] In specific embodiments, the nucleic acids are prepared in vitro from nucleic acids present in, or isolated or partially isolated from a cell culture which are well known in the art, and are described generally, e.g., in Sambrook et al., 2001, *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y.), which is incorporated by reference herein in its entirety.

5.4.1.1 Nucleic Acid Arrays

[0135] In certain embodiments, nucleic acid arrays are employed to generate features of biomarkers in a biomarker profile by detecting the expression of any one or more of the markers described herein. In one embodiment of the invention, a microarray such as a cDNA microarray is used to determine feature values of biomarkers in a biomarker profile. Exemplary methods for cDNA microarray analysis are described below, and in the examples.

[0136] In certain embodiments, the feature values for biomarkers in a biomarker profile are obtained by hybridizing to the array detectably labeled nucleic acids representing or corresponding to the nucleic acid sequences in mRNA transcripts present in a biological sample (e.g., fluorescently labeled cDNA synthesized from the sample) to a microarray comprising one or more probe spots.

[0137] Nucleic acid arrays, for example, microarrays, can be made in a number of ways, of which several are described herein below. Preferably, the arrays are reproducible, allow-

US 2015/0203820 A1

Jul. 23, 2015

11

ing multiple copies of a given array to be produced and results from said microarrays compared with each other. Preferably, the arrays are made from materials that are stable under binding (e.g., nucleic acid hybridization) conditions. Those skilled in the art will know of suitable supports, substrates or carriers for hybridizing test probes to probe spots on an array, or will be able to ascertain the same by use of routine experimentation.

[0138] Arrays, for example, microarrays, used can include one or more test probes. In some embodiments each such test probe comprises a nucleic acid sequence that is complementary to a subsequence of RNA or DNA to be detected. Each probe typically has a different nucleic acid sequence, and the position of each probe on the solid surface of the array is usually known or can be determined. Arrays useful in accordance with the invention can include, for example, oligonucleotide microarrays, cDNA based arrays, SNP arrays, spliced variant arrays and any other array able to provide a qualitative, quantitative or semi-quantitative measurement of expression of a marker described herein. Some types of microarrays are addressable arrays. More specifically, some microarrays are positionally addressable arrays. In some embodiments, each probe of the array is located at a known, predetermined position on the solid support so that the identity (e.g., the sequence) of each probe can be determined from its position on the array (e.g., on the support or surface). In some embodiments, the arrays are ordered arrays. Microarrays are generally described in Draghici, 2003, *Data Analysis Tools for DNA Microarrays*, Chapman & Hall/CRC, which is hereby incorporated herein by reference in its entirety.

5.4.1.2 RT-PCR

[0139] In certain embodiments, to determine the feature values of biomarkers in a biomarker profile of level of expression of one or more of the markers described herein is measured by amplifying RNA from a sample using reverse transcription (RT) in combination with the polymerase chain reaction (PCR). In accordance with this embodiment, the reverse transcription may be quantitative or semi-quantitative. The RT-PCR methods taught herein may be used in conjunction with the microarray methods described above. For example, a bulk PCR reaction may be performed, the PCR products may be resolved and used as probe spots on a microarray.

[0140] Total RNA, or mRNA is used as a template and a primer specific to the transcribed portion of the marker(s) is used to initiate reverse transcription. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook et al., 2001, *supra*. Primer design can be accomplished based on known nucleotide sequences that have been published or available from any publicly available sequence database such as Genbank. For example, primers may be designed for any of the markers described herein. Further, primer design may be accomplished by utilizing commercially available software (e.g., Primer Designer 1.0, Scientific Software etc.). The product of the reverse transcription is subsequently used as a template for PCR.

[0141] PCR provides a method for rapidly amplifying a particular nucleic acid sequence by using multiple cycles of DNA replication catalyzed by a thermostable, DNA-dependent DNA polymerase to amplify the target sequence of interest. PCR requires the presence of a nucleic acid to be amplified, two single-stranded oligonucleotide primers flanking the sequence to be amplified, a DNA polymerase, deoxyribo-

nucleoside triphosphates, a buffer and salts. The method of PCR is well known in the art. PCR, is performed, for example, as described in Mullis and Faloona, 1987, *Methods Enzymol.* 155:335, which is hereby incorporated herein by reference in its entirety.

[0142] PCR can be performed using template DNA or cDNA (at least 1 fg; more usefully, 1-1000 ng) and at least 25 pmol of oligonucleotide primers. A typical reaction mixture includes: 2 .mu.l of DNA, 25 pmol of oligonucleotide primer, 2.5 .mu.l of 10 M PCR buffer 1 (Perkin-Elmer, Foster City, Calif.), 0.4 .mu.l of 1.25 M dNTP, 0.15 .mu.l (or 2.5 units) of Taq DNA polymerase (Perkin Elmer, Foster City, Calif.) and deionized water to a total volume of 25 .mu.l. Mineral oil is overlaid and the PCR is performed using a programmable thermal cycler.

[0143] Quantitative RT-PCR ("QRT-PCR"), which is quantitative in nature, can also be performed to provide a quantitative measure of marker expression levels. In QRT-PCR reverse transcription and PCR can be performed in two steps, or reverse transcription combined with PCR can be performed concurrently. One of these techniques, for which there are commercially available kits such as Taqman (Perkin Elmer, Foster City, Calif.) or as provided by Applied Biosystems (Foster City, Calif.) is performed with a transcript-specific antisense probe. This probe is specific for the PCR product (e.g. a nucleic acid fragment derived from a gene) and is prepared with a quencher and fluorescent reporter probe complexed to the 5' end of the oligonucleotide. Different fluorescent markers are attached to different reporters, allowing for measurement of two products in one reaction. When Taq DNA polymerase is activated, it cleaves off the fluorescent reporters of the probe bound to the template by virtue of its 5'-to-3' exonuclease activity. In the absence of the quenchers, the reporters now fluoresce. The color change in the reporters is proportional to the amount of each specific product and is measured by a fluorometer; therefore, the amount of each color is measured and the PCR product is quantified. The PCR reactions are performed in 96-well plates so that samples derived from many individuals are processed and measured simultaneously. The Taqman system has the additional advantage of not requiring gel electrophoresis and allows for quantification when used with a standard curve.

[0144] A second technique useful for detecting PCR products quantitatively is to use an intercalating dye such as the commercially available QuantiTect SYBR Green PCR (Qiagen, Valencia Calif.). RT-PCR is performed using SYBR green as a fluorescent label which is incorporated into the PCR product during the PCR stage and produces a fluorescence proportional to the amount of PCR product.

[0145] Both Taqman and QuantiTect SYBR systems can be used subsequent to reverse transcription of RNA. Reverse transcription can either be performed in the same reaction mixture as the PCR step (one-step protocol) or reverse transcription can be performed first prior to amplification utilizing PCR (two-step protocol). Additionally, other systems to quantitatively measure mRNA expression products are known including Molecular Beacons® which uses a probe having a fluorescent molecule and a quencher molecule, the probe capable of forming a hairpin structure such that when in the hairpin form, the fluorescence molecule is quenched, and when hybridized the fluorescence increases giving a quantitative measurement of gene expression.

US 2015/0203820 A1

Jul. 23, 2015

12

5.4.1.3 Northern Blot Assays

[0146] Any hybridization technique known to those of skill in the art can be used to generate feature values for biomarkers in a biomarker profile. In other particular embodiments, feature values for biomarkers in a biomarker profile can be obtained by Northern blot analysis (to detect and quantify specific RNA molecules. A standard Northern blot assay can be used to ascertain an RNA transcript size, identify alternatively spliced RNA transcripts, and the relative amounts of one or more genes described herein (in particular, mRNA) in a sample, in accordance with conventional Northern hybridization techniques known to those persons of ordinary skill in the art. In Northern blots, RNA samples are first separated by size via electrophoresis in an agarose gel under denaturing conditions. The RNA is then transferred to a membrane, crosslinked and hybridized with a labeled probe. Nonisotopic or high specific activity radiolabeled probes can be used including random-primed, nick-translated, or PCR-generated DNA probes, in vitro transcribed RNA probes, and oligonucleotides. Additionally, sequences with only partial homology (e.g., cDNA from a different species or genomic DNA fragments that might contain an exon) may be used as probes. The labeled probe, e.g., a radiolabeled cDNA, either containing the full-length, single stranded DNA or a fragment of that DNA sequence may be at least 20, at least 30, at least 50, or at least 100 consecutive nucleotides in length. The probe can be labeled by any of the many different methods known to those skilled in this art. The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals that fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, but are not limited to, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. The radioactive label can be detected by any of the currently available counting procedures. Non-limiting examples of isotopes include .sup.3H, .sup.14C, .sup.32P, .sup.35S, .sup.36Cl, .sup.51Cr, .sup.57Co, .sup.58Co, .sup.59Fe, .sup.90Y, .sup.125I, .sup.131I, and .sup.186Re. Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Any enzymes known to one of skill in the art can be utilized. Examples of such enzymes include, but are not limited to, peroxidase, beta-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Pat. Nos. 3,654,090, 3,850,752, and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

5.4.2 Methods of Detecting Proteins

[0147] In specific embodiments of the invention, feature values of biomarkers in a biomarker profile can be obtained by detecting proteins, for example, by detecting the expression product (e.g., a nucleic acid or protein) of one or more markers described herein, or post-translationally modified, or otherwise modified, or processed forms of such proteins. In a specific embodiment, a biomarker profile is generated by detecting and/or analyzing one or more proteins and/or discriminating fragments thereof expressed from a marker disclosed herein using any method known to those skilled in the

art for detecting proteins including, but not limited to protein microarray analysis, immunohistochemistry and mass spectrometry.

[0148] Standard techniques may be utilized for determining the amount of the protein or proteins of interest present in a cell culture. For example, standard techniques can be employed using, e.g., immunoassays such as, for example Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, (SDS-PAGE), immunocytochemistry, and the like to determine the amount of protein or proteins of interest present in a sample. One exemplary agent for detecting a protein of interest is an antibody capable of specifically binding to a protein of interest, preferably an antibody detectably labeled, either directly or indirectly.

[0149] For such detection methods, if desired a protein from the cell culture to be analyzed can easily be isolated using techniques which are well known to those of skill in the art. Protein isolation methods can, for example, be such as those described in Harlow and Lane, 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y.), which is incorporated by reference herein in its entirety.

[0150] In certain embodiments, methods of detection of the protein or proteins of interest involve their detection via interaction with a protein-specific antibody. For example, antibodies directed to a protein of interest. Antibodies can be generated utilizing standard techniques well known to those of skill in the art. In specific embodiments, antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or an antibody fragment (e.g., scFv, Fab or F(ab').sub.2) can, for example, be used.

[0151] For example, antibodies, or fragments of antibodies, specific for a protein of interest can be used to quantitatively or qualitatively detect the presence of a protein. This can be accomplished, for example, by immunofluorescence techniques. Antibodies (or fragments thereof) can, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of a protein of interest. In situ detection can be accomplished by removing a biological sample (e.g., a biopsy specimen) from a patient, and applying thereto a labeled antibody that is directed to a protein of interest. The antibody (or fragment) is preferably applied by overlaying the antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the protein of interest, but also its distribution, in a particular sample. A wide variety of well-known histological methods (such as staining procedures) can be utilized to achieve such in situ detection.

[0152] Immunoassays for a protein of interest typically comprise incubating a sample of a detectably labeled antibody capable of identifying a protein of interest, and detecting the bound antibody by any of a number of techniques well-known in the art. As discussed in more detail, below, the term "labeled" can refer to direct labeling of the antibody via, e.g., coupling (i.e., physically linking) a detectable substance to the antibody, and can also refer to indirect labeling of the antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody.

[0153] The sample can be brought in contact with and immobilized onto a solid phase support or carrier such as

US 2015/0203820 A1

Jul. 23, 2015

13

nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support can then be washed with suitable buffers followed by treatment with the detectably labeled fingerprint gene-specific antibody. The solid phase support can then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support can then be detected by conventional methods.

[0154] By “solid phase support or carrier” is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material can have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration can be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface can be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

[0155] One of the ways in which an antibody specific for a protein of interest can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, 1978, “The Enzyme Linked Immunosorbent Assay (ELISA)”, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, Md.; Voller et al., 1978, J. Clin. Pathol. 31:507-520; Butler, J. E., 1981, Meth. Enzymol. 73:482-523; Maggio (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, Fla.; Ishikawa et al., (eds.), 1981, Enzyme Immunoassay, Kigaku Shoin, Tokyo, each of which is hereby incorporated by reference in its entirety). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

[0156] Detection can also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect a protein of interest through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, 1986, Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, which is hereby incorporated by reference herein). The radioactive isotope (e.g., .sup.125I, .sup.131I, .sup.35S

or .sup.3H) can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

[0157] It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

[0158] The antibody can also be detectably labeled using fluorescence emitting metals such as .sup.152Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

[0159] The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, therromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

[0160] Likewise, a bioluminescent compound can be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

[0161] In another embodiment, specific binding molecules other than antibodies, such as aptamers, may be used to bind the biomarkers. In yet another embodiment, the biomarker profile may comprise a measurable aspect of an infectious agent (e.g., lipopolysaccharides or viral proteins) or a component thereof.

[0162] In some embodiments, a protein chip assay (e.g., The ProteinChip® Biomarker System, CIPHERgen, Fremont, Calif.) is used to measure feature values for the biomarkers in the biomarker profile. See also, for example, Lin, 2004, Modern Pathology, 1-9; Li, 2004, Journal of Urology 171, 1782-1787; Wadsworth, 2004, Clinical Cancer Research, 10, 1625-1632; Prieto, 2003, Journal of Liquid Chromatography & Related Technologies 26, 2315-2328; Coombes, 2003, Clinical Chemistry 49, 1615-1623; Mian, 2003, Proteomics 3, 1725-1737; Lehre et al., 2003, BJU International 92, 223-225; and Diamond, 2003, Journal of the American Society for Mass Spectrometry 14, 760-765, each of which is hereby incorporated by reference in its entirety.

[0163] In some embodiments, a bead assay is used to measure feature values for the biomarkers in the biomarker profile. One such bead assay is the Becton Dickinson Cytometric Bead Array (CBA). CBA employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes. CBA is combined with flow cytometry to create a multiplexed assay. The Becton Dickinson CBA system, as embodied for example in the Becton Dickinson Human Inflammation Kit, uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate.

US 2015/0203820 A1

Jul. 23, 2015

14

The BD CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample.

[0164] In some embodiments the multiplex analysis method described in U.S. Pat. No. 5,981,180 (“the ‘180 patent”), herein incorporated by reference in its entirety, and in particular for its teachings of the general methodology, bead technology, system hardware and antibody detection, is used to measure feature values for the biomarkers in a biomarker profile. For this analysis, a matrix of microparticles is synthesized, where the matrix consists of different sets of microparticles. Each set of microparticles can have thousands of molecules of a distinct antibody capture reagent immobilized on the microparticle surface and can be color-coded by incorporation of varying amounts of two fluorescent dyes. The ratio of the two fluorescent dyes provides a distinct emission spectrum for each set of microparticles, allowing the identification of a microparticle a set following the pooling of the various sets of microparticles. U.S. Pat. Nos. 6,268,222 and 6,599,331 also are incorporated herein by reference in their entirety, and in particular for their teachings of various methods of labeling microparticles for multiplex analysis.

5.4.3 Use of Other Methods of Detection

[0165] In some embodiments, a separation method may be used to determine feature values for biomarkers in a biomarker profile, such that only a subset of biomarkers within the sample is analyzed. For example, the biomarkers that are analyzed in a sample may be mRNA species from a cellular extract which has been fractionated to obtain only the nucleic acid biomarkers within the sample, or the biomarkers may be from a fraction of the total complement of proteins within the sample, which have been fractionated by chromatographic techniques.

[0166] Feature values for biomarkers in a biomarker profile can also, for example, be generated by the use of one or more of the following methods described below. For example, methods may include nuclear magnetic resonance (NMR) spectroscopy, a mass spectrometry method, such as electrospray ionization mass spectrometry (ESI-MS), ESI-MS/MS, ESI-MS/(MS).sup.n (n is an integer greater than zero), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), desorption/ionization on silicon (DIOS), secondary ion mass spectrometry (SIMS), quadrupole time-of-flight (Q-TOF), atmospheric pressure chemical ionization mass spectrometry (APCI-MS), APCI-MS/MS, APCI-(MS).sup.n, atmospheric pressure photoionization mass spectrometry (APPI-MS), APPI-MS/MS, and APPI-(MS).sup.n. Other mass spectrometry methods may include, inter alia, quadrupole, Fourier transform mass spectrometry (FTMS) and ion trap. Other suitable methods may include chemical extraction partitioning, column chromatography, ion exchange chromatography, hydrophobic (reverse phase) liquid chromatography, isoelectric focusing, one-dimensional polyacrylamide gel electrophoresis (PAGE), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or other chromatography, such as thin-layer, gas or liquid chromatography, or any combination thereof. In one embodiment, the biological sample may be fractionated prior to application of the separation method.

[0167] In one embodiment, laser desorption/ionization time-of-flight mass spectrometry is used to create determine

feature values in a biomarker profile where the biomarkers are proteins or protein fragments that have been ionized and vaporized off an immobilizing support by incident laser radiation and the feature values are the presence or absence of peaks representing these fragments in the mass spectra profile. A variety of laser desorption/ionization techniques are known in the art (see, e.g., Guttman et al., 2001, Anal. Chem. 73:1252-62 and Wei et al., 1999, Nature 399:243-246, each of which is hereby incorporated by herein be reference in its entirety).

[0168] Laser desorption/ionization time-of-flight mass spectrometry allows the generation of large amounts of information in a relatively short period of time. A biological sample is applied to one of several varieties of a support that binds all of the biomarkers, or a subset thereof, in the sample. Cell lysates or samples are directly applied to these surfaces in volumes as small as 0.5 μ L, with or without prior purification or fractionation. The lysates or sample can be concentrated or diluted prior to application onto the support surface. Laser desorption/ionization is then used to generate mass spectra of the sample, or samples, in as little as three hours.

5.4.4 Data Analysis Algorithms

[0169] Biomarker expression profile of hES-MSC are factors discriminating between clinical grade hES-MSC and non-clinical grade hES-MSC. The identity of these biomarkers and their corresponding features (e.g., expression levels) can be used to develop a decision rule, or plurality of decision rules, that discriminate between clinical grade and non-clinical grade hES-MSC. Specific data analysis algorithms for building a decision rule, or plurality of decision rules, that discriminate between clinical grade hES-MSC and non-clinical grade hES-MSC. Once a decision rule has been built using these exemplary data analysis algorithms or other techniques known in the art, the decision rule can be used to classify a hES-MSC population into one of the two or more phenotypic classes (e.g. a clinical grade or a non-clinical grade hES-MSC). This is accomplished by applying the decision rule to a biomarker profile obtained from the cell culture. Such decision rules, therefore, have enormous value as defining the quality of hES-MSC. In certain embodiment, provided herein is a method for the evaluation of a biomarker profile from a test cell culture to biomarker profiles obtained from a cell culture in a control population. In some embodiments, each biomarker profile obtained from the control population, as well as the test cell culture, comprises a feature for each of a plurality of different biomarkers. In some embodiments, this comparison is accomplished by (i) developing a decision rule using the biomarker profiles from the control population and (ii) applying the decision rule to the biomarker profile from the test cell culture. As such, the decision rules applied in some embodiments of the present invention are used to determine whether a test cell culture is clinical grade or non-clinical grade. In certain embodiment, the control population is a clinical grade hES-MSC. In another embodiment, the control population is BM-MSC.

[0170] In some embodiments of the present invention, when the results of the application of a decision rule indicate that the test cell culture is clinical grade hES-MSC, it is used for treatment. If the results of an application of a decision rule indicate that the test cell culture is non-clinical grade hES-MSC, the test cell culture is not used for treatment.

US 2015/0203820 A1

Jul. 23, 2015

15

5.5 Modification of MSC

[0171] Provided herein is a method of modifying mesenchymal stem cells to produce a population of modified MSC that has improved immunosuppressive function. The MSC have the following characteristics: (i) contain >95% of cells expressing group-1 markers; (ii) contain >80% of cells expressing group 2 markers; (iii) contain <5% of cells expressing group-3 markers (iv) expressing IL-10 and TGF β ; (v) contain <2% of cells expressing IL-6, IL-12 and TNF α ; and (vi) contains <0.001% of cells co-expressing all group-4 markers, wherein group-1 markers are CD73, CD90, CD105, CD146, CD166, and CD44, group-2 markers are CD13, CD29, CD54, CD49E, group-3 markers are CD45, CD34, CD31 and SSEA4, and group-4 markers are OCT4, NANOG, TRA-1-60 and SSEA4.

[0172] Provided herein is a method of increasing immunosuppressive function of hES-MSC by increasing the expression of AIF. In an embodiment, the method comprises decreasing the expression of PLF. In an embodiment, the method comprises decreasing the expression of IL6, IL12, TNF α , RAGE and other PIF in hES-MSC. In an embodiment, the method comprises increasing the expression of TGF β and IL-10 in hES-MSC.

[0173] In certain embodiments, the method comprises genetic and epigenetic modification of hES-MSC that are known in the art. In certain embodiments, the genetic modification or epigenetic regulation includes, but are not limited to, knockout, small hair pin RNA ("shRNA"), micro RNA ("miRNA"), non-coding RNA ("ncRNA"), morpholino oligo, decoy RNA, DNA methylation regulation, histone methylation regulation, translation inhibition and/or antibody blocking. In certain embodiment, MSC are modified through transposomes, toll-like receptor ligands, small molecules.

[0174] In certain embodiments, small molecules are used to target any of the signaling pathway components of IL-6 signaling. In certain embodiments, the target includes, but not limited to, gp130, STAT3, Cathepsin S, NFkappaB, IRF5. In certain embodiments, IL-12 expression is decreased in hES-MSC by activation of the prostaglandin E2 pathway, by increasing intracellular cyclic AMP levels with cAMP agonists that include but are not limited to forskolin, cholera toxin, β 1- and β 2 adrenoreceptor agonists, by inhibition of the NF- κ B Rel-B pathway, by treating hES-MSC with apoptotic cells, by treatment with phosphatidylserine, by treatment with butyrate, by treatment with Triptolide or extracts from *Tripterygium wilfordii* or synthetic forms or Triptolide (i.e. Minnelide).

[0175] In certain embodiments, MSC may be modified to express a certain marker using methods known in the art of recombinant DNA. In certain embodiment, MSC may be modified by transfection using the nucleotide sequence encoding the marker. The marker can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The necessary transcriptional and translational elements can also be present. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. A variety of host-vector systems may be utilized to express the marker. These include, but are not limited to, mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA;

and stable cell lines generated by transformation using a selectable marker. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

[0176] Once a vector encoding the appropriate marker has been synthesized, the MSC is transformed or transfected with the vector of interest.

[0177] Standard methods of introducing a nucleic acid sequence of interest into the MSC can be used. Transformation may be by any known method for introducing polynucleotides into a host cell, including, for example packaging the polynucleotide in a virus and transducing a host cell with the virus, and by direct uptake of the polynucleotide. Mammalian transformations (i.e., transfections) by direct uptake may be conducted using the calcium phosphate precipitation method of Graham & Van der Eb, 1978, *Virol.* 52:546, or the various known modifications thereof. Other methods for introducing recombinant polynucleotides into cells, particularly into mammalian cells, include dextran-mediated transfection, calcium phosphate mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct micro-injection of the polynucleotides into nuclei. Such methods are well-known to one of skill in the art.

[0178] In a preferred embodiment, stable cell lines containing the constructs of interest are generated for high throughput screening. Such stable cells lines may be generated by introducing a construct comprising a selectable marker, allowing the cells to grow for 1-2 days in an enriched medium, and then growing the cells on a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

[0179] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, *Cell* 22:817) genes can be employed in tk-, hgprt- or apt- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, *Natl. Acad. Sci. USA* 77:3567; O'Hare, et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, *J. Mol. Biol.* 150:1); and hygromycin (Santerre, et al., 1984, *Gene* 30:147) genes.

5.6 Stem Cell Collection Composition

[0180] The stem cell collection composition can comprise any physiologically-acceptable solution suitable for the collection and/or culture of stem cells, for example, a saline solution (e.g., phosphate-buffered saline, Krebs's solution, modified Krebs's solution, Eagle's solution, 0.9% NaCl, etc.), a culture medium (e.g., DMEM, H. DMEM, etc.), and the like.

[0181] The stem cell collection composition can comprise one or more components that tend to preserve stem cells, that is, prevent the stem cells from dying, or delay the death of the

US 2015/0203820 A1

Jul. 23, 2015

16

stem cells, reduce the number of stem cells in a population of cells that die, or the like, from the time of collection to the time of culturing. Such components can be, e.g., an apoptosis inhibitor (e.g., a caspase inhibitor or JNK inhibitor); a vasodilator (e.g., magnesium sulfate, an antihypertensive drug, atrial natriuretic peptide (ANP), adrenocorticotropin, corticotropin-releasing hormone, sodium nitroprusside, hydralazine, adenosine triphosphate, adenosine, indomethacin or magnesium sulfate, a phosphodiesterase inhibitor, etc.); a necrosis inhibitor (e.g., 2-(1H-Indol-3-yl)-3-pentylamino-maleimide, pyrrolidine dithiocarbamate, or clonazepam); a TNF- α inhibitor; and/or an oxygen-carrying perfluorocarbon (e.g., perfluorooctyl bromide, perfluorodecyl bromide, etc.).

[0182] The stem cell collection composition can comprise one or more tissue-degrading enzymes, e.g., a metalloprotease, a serine protease, a neutral protease, an RNase, or a DNase, or the like. Such enzymes include, but are not limited to, collagenases (e.g., collagenase I, II, III or IV, a collagenase from *Clostridium histolyticum*, etc.); dispase, thermolysin, elastase, trypsin, LIBERASE, hyaluronidase, and the like.

[0183] The stem cell collection composition can comprise a bacteriostatically or bacteriostatically effective amount of an antibiotic. In certain non-limiting embodiments, the antibiotic is a macrolide (e.g., tobramycin), a cephalosporin (e.g., cephalixin, cephradine, cefuroxime, cefprozil, cefaclor, cefixime or cefadroxil), a clarithromycin, an erythromycin, a penicillin (e.g., penicillin V) or a quinolone (e.g., ofloxacin, ciprofloxacin or norfloxacin), a tetracycline, a streptomycin, etc. In a particular embodiment, the antibiotic is active against Gram(+) and/or Gram(-) bacteria, e.g., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and the like.

[0184] The stem cell collection composition can also comprise one or more of the following compounds: adenosine (about 1 mM to about 50 mM); D-glucose (about 20 mM to about 100 mM); magnesium ions (about 1 mM to about 50 mM); a macromolecule of molecular weight greater than 20,000 daltons, in one embodiment, present in an amount sufficient to maintain endothelial integrity and cellular viability (e.g., a synthetic or naturally occurring colloid, a polysaccharide such as dextran or a polyethylene glycol present at about 25 g/l to about 100 g/l, or about 40 g/l to about 60 g/l); an antioxidant (e.g., butylated hydroxyanisole, butylated hydroxytoluene, glutathione, vitamin C or vitamin E present at about 25 μ M to about 100 μ M); a reducing agent (e.g., N-acetylcysteine present at about 0.1 mM to about 5 mM); an agent that prevents calcium entry into cells (e.g., verapamil present at about 2 μ M to about 25 μ M); nitroglycerin (e.g., about 0.05 g/L to about 0.2 g/L); an anticoagulant, in one embodiment, present in an amount sufficient to help prevent clotting of residual blood (e.g., heparin or hirudin present at a concentration of about 1000 units/l to about 100,000 units/l); or an amiloride containing compound (e.g., amiloride, ethyl isopropyl amiloride, hexamethylene amiloride, dimethyl amiloride or isobutyl amiloride present at about 1.0 μ M to about 5 μ M).

5.7 Immunomodulation Using hES-MSC or iPS-MSC

[0185] Provided herein is the modulation of the activity (e.g. reduced cell proliferation, reduced cell survival, impaired cell migration to sites of inflammation, reduced ability of the cells to promote or prolong inflammation or enhanced cell functions that promote the restoration of healthy tissue or organ homeostasis) of an immune cell, or

plurality of immune cells, by contacting the immune cell(s) with a plurality of hES-MSC or iPS-MSC. In one embodiment, the method of modulating an immune response comprises contacting a plurality of immune cells with a plurality of hES-MSC or iPS-MSC for a time sufficient for said hES-MSC or iPS-MSC to detectably suppress an immune response, wherein said hES-MSC or iPS-MSC detectably suppress T cell proliferation in a mixed lymphocyte reaction (MLR) assay.

[0186] Since BM-MSC or other adult tissue derived MSC has been used to treat many autoimmune disease, BM-MSC is also used for tissue repairing by limit inflammation and secret growth and protective factors, replace damaged tissues. We have showed here in our examples that hES-MSC have superior immunosuppressive function than BM-MSC, thus hES-MSC can be used in all areas and diseases that currently targeted by BM-MSC.

[0187] hES-MSC or iPS-MSC used for immunomodulation may be derived or obtained from an embryonic stem cell line or induced pluripotent stem cell line, respectively, hES-MSC or iPS-MSC used for immunomodulation may also be derived from a the same species as the immune cells whose activity is to be modulated or from a different species as that of the immune cells whose activity is to be modulated.

[0188] An “immune cell” in the context of this method means any cell of the immune system, particularly T cells and NK (natural killer) cells. Thus, in various embodiments of the method, hES-MSC are contacted with a plurality of immune cells, wherein the plurality of immune cells are, or comprises, a plurality of T cells (e.g., a plurality of CD3.sup.+ T cells, CD4.sup.+ T cells and/or CD8.sup.+ T cells) and/or natural killer cells. An “immune response” in the context of the method can be any response by an immune cell to a stimulus normally perceived by an immune cell, e.g., a response to the presence of an antigen. In various embodiments, an immune response can be the proliferation of T cells (e.g., CD3.sup.+ T cells, CD4.sup.+ T cells and/or CD8.sup.+ T cells) in response to a foreign antigen, such as an antigen present in a transfusion or graft, or to a self-antigen, as in an autoimmune disease. The immune response can also be a proliferation of T cells contained within a graft. The immune response can also be any activity of a natural killer (NK) cell, the maturation of a dendritic cell, or the like. The immune response can also be a local, tissue- or organ-specific, or systemic effect of an activity of one or more classes of immune cells, e.g. the immune response can be graft versus host disease, inflammation, formation of inflammation-related scar tissue, an autoimmune condition (e.g., rheumatoid arthritis, Type I diabetes, lupus erythematosus, etc.) and the like.

[0189] “Contacting” in this context encompasses bringing the hES-MSC and immune cells together in a single container (e.g., culture dish, flask, vial, etc.) or in vivo, for example, the same individual (e.g., mammal, for example, human). In a preferred embodiment, the contacting is for a time sufficient, and with a sufficient number of hES-MSC and immune cells, that a change in an immune function of the immune cells is detectable. More preferably, in various embodiments, said contacting is sufficient to suppress immune function (e.g., T cell proliferation in response to an antigen) by at least 50%, 60%, 70%, 80%, 90% or 95%, compared to the immune function in the absence of the hES-MSC. Such suppression in an in vivo context can be determined in an in vitro assay; that is, the degree of suppression in the in vitro assay can be extrapolated, for a particular number of hES-MSC and a

US 2015/0203820 A1

Jul. 23, 2015

17

number of immune cells in a recipient individual, to a degree of suppression in the individual.

[0190] The invention in certain embodiments provides methods of using hES-MSC to modulate an immune response, or the activity of a plurality of one or more types of immune cells, *in vitro*. Contacting the hES-MSC and plurality of immune cells can comprise combining the hES-MSC and immune cells in the same physical space such that at least a portion of the plurality of hES-MSC interacts with at least a portion of the plurality of immune cells; maintaining the hES-MSC and immune cells in separate physical spaces with common medium; or can comprise contacting medium from one or a culture of hES-MSC or immune cells with the other type of cell (for example, obtaining culture medium from a culture of hES-MSC and resuspending isolated immune cells in the medium). In a specific example, the contacting is a Mixed Lymphocyte Reaction (MLR).

[0191] Such contacting can, for example, take place in an experimental setting designed to determine the extent to which a particular plurality of hES-MSC is immunomodulatory, e.g., immunosuppressive. Such an experimental setting can be, for example, a mixed lymphocyte reaction (MLR) or regression assay. Procedures for performing the MLR and regression assays are well-known in the art. See, e.g. Schwarz, "The Mixed Lymphocyte Reaction: An In Vitro Test for Tolerance." *J. Exp. Med.* 127(5):879-890 (1968); Lacerda et al., "Human Epstein-Barr Virus (EBV)-Specific Cytotoxic T Lymphocytes Home Preferentially to and Induce Selective Regressions of Autologous EBV-Induced B Lymphoproliferations in Xenografted C.B-17 Scid/Scid Mice," *J. Exp. Med.* 183:1215-1228 (1996). In a preferred embodiment, an MLR is performed in which a plurality of hES-MSC are contacted with a plurality of immune cells (e.g., lymphocytes, for example, CD3⁺ CD4⁺ and/or CD8⁺ T lymphocytes).

[0192] The MLR can be used to determine the immunosuppressive capacity of a plurality of hES-MSC. For example, a plurality of hES-MSC can be tested in an MLR comprising combining CD4⁺ or CD8⁺ T cells, dendritic cells (DC) and hES-MSC in a ratio of about 10:1:2, wherein the T cells are stained with a dye such as, e.g., CFSE that partitions into daughter cells, and wherein the T cells are allowed to proliferate for about 6 days. The plurality of hES-MSC is immunosuppressive if the T cell proliferation at 6 days in the presence of hES-MSC is detectably reduced compared to T cell proliferation in the presence of DC and absence of hES-MSC. In such an MLR, hES-MSC are either thawed or harvested from culture. About 20,000 hES-MSC are resuspended in 100 μ l of medium (RPMI 1640, 1 mM HEPES buffer, antibiotics, and 5% pooled human serum), and allowed to attach to the bottom of a well for 2 hours. CD4 and/or CD8 T cells are isolated from whole peripheral blood mononuclear cells Miltenyi magnetic beads. The cells are CFSE stained, and a total of 100,000 T cells (CD4⁺ T cells alone, CD8⁺ T cells alone, or equal amounts of CD4⁺ and CD8⁺ T cells) are added per well. The volume in the well is brought to 200 μ l, and the MLR is allowed to proceed.

[0193] In one embodiment, therefore, the invention provides a method of suppressing an immune response comprising contacting a plurality of immune cells with a plurality of hES-MSC for a time sufficient for said hES-MSC to detectably suppress T cell proliferation in a mixed lymphocyte reaction (MLR) assay.

[0194] Populations of hES-MSC obtained from different embryonic stem cell line, can differ in their ability to modu-

late an activity of an immune cell, e.g., can differ in their ability to suppress T cell activity or proliferation or NK cell activity. It is thus desirable to determine, prior to use, the capacity of a particular population of hES-MSC for immunosuppression. Such a capacity can be determined, for example, by testing a sample of the stem cell population in an MLR or regression assay. In one embodiment, an MLR is performed with the sample, and a degree of immunosuppression in the assay attributable to the hES-MSC is determined. This degree of immunosuppression can then be attributed to the stem cell population that was sampled. Thus, the MLR can be used as a method of determining the absolute and relative ability of a particular population of hES-MSC to suppress immune function. The parameters of the MLR can be varied to provide more data or to best determine the capacity of a sample of hES-MSC to immunosuppress. For example, because immunosuppression by hES-MSC appears to increase roughly in proportion to the number of hES-MSC present in the assay, the MLR can be performed with, in one embodiment, two or more numbers of stem cells, e.g., 1×10^3 , 3×10^3 , 1×10^4 and/or 3×10^4 hES-MSC per reaction. The number of hES-MSC relative to the number of T cells in the assay can also be varied. For example, hES-MSC and T cells in the assay can be present in any ratio of, e.g. about 10:1 to about 1:10, preferably about 1:5, though a relatively greater number of hES-MSC or T cells can be used.

[0195] The invention also provides methods of using hES-MSC to modulate an immune response, or the activity of a plurality of one or more types of immune cells, *in vivo*. hES-MSC and immune cells can be contacted, e.g., in an individual that is a recipient of a plurality of hES-MSC. Where the contacting is performed in an individual, in one embodiment, the contacting is between exogenous hES-MSC (that is, hES-MSC not derived from the individual) and a plurality of immune cells endogenous to the individual. In specific embodiments, the immune cells within the individual are CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, and/or NK cells.

[0196] Such immunosuppression using hES-MSC would be advantageous for any condition caused or worsened by, or related to, an inappropriate or undesirable immune response. hES-MSC-mediated immunomodulation, e.g., immunosuppression, would, for example, be useful in the suppression of an inappropriate immune response raised by the individual's immune system against one or more of its own tissues. In various embodiments, therefore, the invention provides a method of suppressing an immune response, wherein the immune response is an autoimmune disease, e.g., lupus erythematosus, diabetes, rheumatoid arthritis, or multiple sclerosis.

[0197] The contacting of the plurality of hES-MSC with the plurality of one or more types of immune cells can occur *in vivo* in the context of, or as an adjunct to, for example, grafting or transplanting of one or more types of tissues to a recipient individual. Such tissues may be, for example, bone marrow or blood; an organ; a specific tissue (e.g., skin graft); composite tissue allograft (i.e., a graft comprising two or more different types of tissues); etc. In this regard, the hES-MSC can be used to suppress one or more immune responses of one or more immune cells contained within the recipient individual, within the transplanted tissue or graft, or both. The contacting can occur before, during and/or after the grafting or transplanting. For example, hES-MSC can be administered at the time of the transplant or graft. The hES-MSC can also,

US 2015/0203820 A1

Jul. 23, 2015

18

or alternatively, be administered prior to the transplanting or grafting, e.g., about 1, 2, 3, 4, 5, 6 or 7 days prior to the transplanting or grafting, hES-MSC can also, or alternatively, be administered to a transplant or graft recipient after the transplantation or grafting, for example, about 1, 2, 3, 4, 5, 6 or 7 days after the transplanting or grafting. Preferably, the plurality of hES-MSC are contacted with the plurality of hES-MSC before any detectable sign or symptom of an immune response, either by the recipient individual or the transplanted tissue or graft, e.g., a detectable sign or symptom of graft-versus-host disease or detectable inflammation, is detectable.

[0198] In another embodiment, the contacting within an individual is primarily between exogenous hES-MSC and exogenous progenitor cells or stem cells, e.g., exogenous progenitor cells or stem cells that differentiate into immune cells. For example, individuals undergoing partial or full immunoablation or myeloablation as an adjunct to cancer therapy can receive hES-MSC in combination with one or more other types of stem or progenitor cells. For example, the hES-MSC can be combined with a plurality of CD34⁺ cells, e.g., CD34⁺ hematopoietic stem cells. Such CD34⁺ cells can be, e.g., CD34⁺ cells from a tissue source such as peripheral blood, umbilical cord blood, placental blood, or bone marrow. The CD34⁺ cells can be isolated from such tissue sources, or the whole tissue source (e.g., units of umbilical cord blood or bone marrow) or a partially purified preparation from the tissue source (e.g., white blood cells from cord blood) can be combined with the hES-MSC.

[0199] The hES-MSC are administered to the individual preferably in a ratio, with respect to the known or expected number of immune cells, e.g., T cells, in the individual, of from about 10:1 to about 1:10, preferably about 1:5. However, a plurality of hES-MSC can be administered to an individual in a ratio of in non-limiting examples, about 10,000:1, about 1,000:1, about 100:1, about 10:1, about 1:1, about 1:10, about 1:100, about 1:1,000 or about 1:10,000. Generally, about 1×10^5 to about 1×10^8 hES-MSC per recipient kilogram, preferably about 1×10^6 to about 1×10^7 hES-MSC recipient kilogram can be administered to effect immunosuppression. In various embodiments, a plurality of hES-MSC administered to an individual or subject comprises at least, about, or no more than, 1×10^5 , 3×10^5 , 1×10^6 , 3×10^6 , 1×10^7 , 3×10^7 , 1×10^8 , 3×10^8 , 1×10^9 , 3×10^9 hES-MSC, or more.

[0200] The hES-MSC can also be administered with one or more second types of stem cells, e.g., mesenchymal stem cells from bone marrow. Such second stem cells can be administered to an individual with hES-MSC in a ratio of, e.g., about 1:10 to about 10:1.

[0201] To facilitate contacting the hES-MSC and immune cells in vivo, the hES-MSC can be administered to the individual by any route sufficient to bring the hES-MSC and immune cells into contact with each other. For example, the hES-MSC can be administered to the individual, e.g., intravenously, intramuscularly, intraperitoneally, or directly into an organ, e.g., pancreas. For in vivo administration, the hES-MSC can be formulated as a pharmaceutical composition.

[0202] The method of immunosuppression can additionally comprise the addition of one or more immunosuppressive agents, particularly in the in vivo context. In one embodiment, the plurality of hES-MSC are contacted with the plurality of immune cells in vivo in an individual, and a composition comprising an immunosuppressive agent is administered to the individual. Immunosuppressive agents are well-known in

the art and include, e.g., anti-T cell receptor antibodies (monoclonal or polyclonal, or antibody fragments or derivatives thereof), anti-IL-2 receptor antibodies (e.g., Basiliximab (SIMULACT®) or daclizumab (ZENAPAX®)), anti T cell receptor antibodies (e.g., Muromonab-CD3), azathioprine, corticosteroids, cyclosporine, tacrolimus, mycophenolate mofetil, sirolimus, calcineurin inhibitors, and the like. In a specific embodiment, the immunosuppressive agent is a neutralizing antibody to macrophage inflammatory protein (MIP)-1 α or MIP-1 β .

5.8 Preservation of hES-MSC

[0203] hES-MSC can be preserved, that is, placed under conditions that allow for long-term storage, or conditions that inhibit cell death by, e.g., apoptosis or necrosis. hES-MSC can be preserved using, e.g., a composition comprising an apoptosis inhibitor, necrosis inhibitor. In one embodiment, the invention provides a method of preserving a population of stem cells comprising contacting said population of stem cells with a stem cell collection composition comprising an inhibitor of apoptosis, wherein said inhibitor of apoptosis is present in an amount and for a time sufficient to reduce or prevent apoptosis in the population of stem cells, as compared to a population of stem cells not contacted with the inhibitor of apoptosis. In a specific embodiment, said inhibitor of apoptosis is a caspase-3 inhibitor. In another specific embodiment, said inhibitor of apoptosis is a JNK inhibitor. In a more specific embodiment, said JNK inhibitor does not modulate differentiation or proliferation of said stem cells. In another embodiment, said stem cell collection composition comprises said inhibitor of apoptosis and said oxygen-carrying perfluorocarbon in separate phases. In another embodiment, said stem cell collection composition comprises said inhibitor of apoptosis and said oxygen-carrying perfluorocarbon in an emulsion. In another embodiment, the stem cell collection composition additionally comprises an emulsifier, e.g., lecithin. In another embodiment, said apoptosis inhibitor and said perfluorocarbon are between about 0° C. and about 25° C. at the time of contacting the stem cells. In another more specific embodiment, said apoptosis inhibitor and said perfluorocarbon are between about 2° C. and 10° C., or between about 2° C. and about 5° C., at the time of contacting the stem cells. In another more specific embodiment, said contacting is performed during transport of said population of stem cells. In another more specific embodiment, said contacting is performed during freezing and thawing of said population of stem cells.

[0204] In certain embodiments, rock-inhibitor Y27632 may be added as a preservation helper. In certain embodiments, the rock-inhibitor is added at concentration of 10 μ M.

[0205] In another embodiment, the invention provides a method of preserving a population of hES-MSC comprising contacting said population of stem cells with an inhibitor of apoptosis and an organ-preserving compound, wherein said inhibitor of apoptosis is present in an amount and for a time sufficient to reduce or prevent apoptosis in the population of stem cells, as compared to a population of stem cells not contacted with the inhibitor of apoptosis.

[0206] Typically, during hES-MSC collection, enrichment and isolation, it is preferable to minimize or eliminate cell stress due to hypoxia and mechanical stress. In another embodiment of the method, therefore, a stem cell, or population of stem cells, is exposed to a hypoxic condition during collection, enrichment or isolation for less than six hours

US 2015/0203820 A1

Jul. 23, 2015

19

during said preservation, wherein a hypoxic condition is a concentration of oxygen that is less than normal blood oxygen concentration. In a more specific embodiment, said population of stem cells is exposed to said hypoxic condition for less than two hours during said preservation. In another more specific embodiment, said population of stem cells is exposed to said hypoxic condition for less than one hour, or less than thirty minutes, or is not exposed to a hypoxic condition, during collection, enrichment or isolation. In another specific embodiment, said population of stem cells is not exposed to shear stress during collection, enrichment or isolation.

[0207] The HES-MS-C can be cryopreserved, e.g., in cryopreservation medium in small containers, e.g., ampoules. Suitable cryopreservation medium includes, but is not limited to, culture medium including, e.g., growth medium, or cell freezing medium, for example commercially available cell freezing medium, e.g., C2695, C2639 or C6039 (Sigma). Cryopreservation medium preferably comprises DMSO (dimethylsulfoxide), at a concentration of, e.g., about 5-10% (v/v). Cryopreservation medium may comprise additional agents, for example, methylcellulose and/or glycerol. HES-MS-C are preferably cooled at about 1° C./min during cryopreservation. A preferred cryopreservation temperature is about -80° C. to about -180° C., preferably about -125° C. to about -140° C. Cryopreserved cells can be transferred to liquid nitrogen prior to thawing for use. In some embodiments, for example, once the ampoules have reached about -90° C., they are transferred to a liquid nitrogen storage area. Cryopreserved cells preferably are thawed at a temperature of about 25° C. to about 40° C., preferably to a temperature of about 37° C.

5.9 Cryopreserved hES-MS-C

[0208] The hES-MS-C disclosed herein can be preserved, for example, cryopreserved for later use. Methods for cryopreservation of cells, such as stem cells, are well known in the art. hES-MS-C can be prepared in a form that is easily administrable to an individual. For example, provided herein are hES-MS-C that are contained within a container that is suitable for medical use. Such a container can be, for example, a sterile plastic bag, flask, jar, or other container from which the hES-MS-C can be easily dispensed. For example, the container can be a blood bag or other plastic, medically-acceptable bag suitable for the intravenous administration of a liquid to a recipient. The container is preferably one that allows for cryopreservation of the combined stem cell population. Cryopreserved hES-MS-C can comprise hES-MS-C derived from a single donor, or from multiple donors. The hES-MS-C can be completely HLA-matched to an intended recipient, or partially or completely HLA-mismatched.

[0209] In another specific embodiment, the container is a bag, flask, or jar. In more specific embodiment, said bag is a sterile plastic bag. In a more specific embodiment, said bag is suitable for, allows or facilitates intravenous administration of the hES-MS-C. The bag can comprise multiple lumens or compartments that are interconnected to allow mixing of the hES-MS-C and one or more other solutions, e.g., a drug, prior to, or during, administration. In another specific embodiment, the composition comprises one or more compounds that facilitate cryopreservation of the combined stem cell population. In another specific embodiment, said hES-MS-C is contained within a physiologically-acceptable aqueous solution. In a more specific embodiment, said physiologically-acceptable aqueous solution is a 0.9% NaCl solution. In another

specific embodiment, said hES-MS-C are HLA-matched to a recipient of said stem cell population. In another specific embodiment, said combined stem cell population comprises hES-MS-C that are at least partially HLA-mismatched to a recipient of said stem cell population.

5.10 Pharmaceutical Preparations

[0210] As discussed above, one embodiment of the present invention is a pharmaceutical composition comprising a therapeutically effective amount of the human embryonic stem cell derived mesenchymal stem cells and a pharmaceutically acceptable carrier. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human, and approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. "Carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

[0211] These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations, cachets, troches, lozenges, dispersions, suppositories, ointments, cataplasms (poultices), pastes, powders, dressings, creams, plasters, patches, aerosols, gels, liquid dosage forms suitable for parenteral administration to a patient, and sterile solids (e.g., crystalline or amorphous solids) that can be reconstituted to provide liquid dosage forms suitable for parenteral administration to a patient. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable form of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[0212] Pharmaceutical compositions adapted for oral administration may be capsules, tablets, powders, granules, solutions, syrups, suspensions (in non-aqueous or aqueous liquids), or emulsions. Tablets or hard gelatin capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, stearic acid or salts thereof. Soft gelatin capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols. Solutions and syrups may comprise water, polyols, and sugars. An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract. Thus, the sustained release may be achieved over many hours and if necessary, the active agent

US 2015/0203820 A1

Jul. 23, 2015

20

can be protected from degradation within the stomach. Pharmaceutical compositions for oral administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

[0213] Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient over a prolonged period of time.

[0214] Pharmaceutical compositions adapted for nasal and pulmonary administration may comprise solid carriers such as powders which can be administered by rapid inhalation through the nose. Compositions for nasal administration may comprise liquid carriers, such as sprays or drops. Alternatively, inhalation directly through into the lungs may be accomplished by inhalation deeply or installation through a mouthpiece. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient.

[0215] Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain anti-oxidants, buffers, bacteriostats, and solutes that render the compositions substantially isotonic with the blood of the subject. Other components which may be present in such compositions include water, alcohols, polyols, glycerine, and vegetable oils. Compositions adapted for parental administration may be presented in unit-dose or multi-dose containers, such as sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile carrier, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets. Suitable vehicles that can be used to provide parenteral dosage forms of the invention are well known to those skilled in the art. Examples include: Water for Injection USP; aqueous vehicles such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as ethyl alcohol, polyethylene glycol, and polypropylene glycol; and non-aqueous vehicles such as corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

[0216] Selection of a therapeutically effective dose will be determined by the skilled artisan considering several factors which will be known to one of ordinary skill in the art. Such factors include the particular form of the inhibitor, and its pharmacokinetic parameters such as bioavailability, metabolism, and half-life, which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether the administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus, the precise dose should be decided according to the judgment of the person of skill in the art, and each patient's circumstances, and according to standard clinical techniques.

[0217] In certain embodiments, patients are treated with antipyretic and/or antihistamine (acetaminophen and diphenhydramine hydrochloride) to minimize any possible DMSO infusion toxicity related to the cryopreserve component in the hES-MSC treatment.

5.11 hES-MSC Conditioned Medium and Derivatives

[0218] The hES-MSC disclosed herein can be used to produce conditioned medium, concentrate of conditioned medium, cell lysate or other derivatives that is immunosuppressive, that is, medium comprising one or more biomolecules secreted or excreted by the stem cells that have a detectable immunosuppressive effect on a plurality of one or more types of immune cells. In various embodiments, the conditioned medium comprises medium in which hES-MSC have grown for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more days. In other embodiments, the conditioned medium comprises medium in which hES-MSC have grown to at least 30%, 40%, 50%, 60%, 70%, 80%, 90% confluence, or up to 100% confluence. Such conditioned medium can be used to support the culture of a separate population of hES-MSC, or stem cells of another kind. In another embodiment, the conditioned medium comprises medium in which hES-MSC have been differentiated into an adult cell type. In another embodiment, the conditioned medium of the invention comprises medium in which hES-MSC and non-hES-MSC have been cultured. In various embodiments, cell lysate comprises of hES-MSC cells lysed by repeat frozen-thaw procedures, or by hypertonic or hypotonic solution treatment.

[0219] Thus, in one embodiment, the invention provides a composition comprising culture medium, cell lysate and/or other derivatives from a culture of hES-MSC, wherein said hES-MSC (a) adhere to a substrate; (b) express CD73, CD105, CD90, CD29, CD44, CD146, IL-10, TGF β 2, HGF, but do not express IL-6, TNF α , IL-12 and/or RAGE. In another specific embodiment, the composition comprises an anti-proliferative agent, e.g., an anti-MIP-1 α or anti-MIP-1 β antibody.

[0220] Provided herein is a method of using hES-MSC as described herein as feeder cells for bone marrow hematopoietic stem cell, peripheral blood hematopoietic stem cell and umbilical-cord hematopoietic stem cell expansion. In certain embodiments, the hES-MSC suitable for the disclosed method express Stro-3, Stro-1, DL1, and/or Nestin. In certain embodiment, hES-MSC is co-cultured with bone marrow hematopoietic stem cells, peripheral blood hematopoietic stem cells and/or umbilical-cord hematopoietic stem cells. In certain embodiment, the hES-MSC is mesenchymal stromal cells. Provided herein is a co-culture of hES-MSC as described herein and bone marrow hematopoietic stem cells. Provided herein is a co-culture of hES-MSC as described herein and umbilical-cord hematopoietic stem cells.

5.12 Matrices Comprising hES-MSC

[0221] The invention further comprises matrices, hydrogels, scaffolds, and the like that comprise hES-MSC. hES-MSC can be seeded onto a natural matrix. e.g., a biomaterial. In certain embodiments, the scaffold is obtained by 3D printing. The hES-MSC can be suspended in a hydrogel solution suitable for, e.g., injection. Suitable hydrogels for such compositions include self-assembling peptides, such as RAD16. In one embodiment, a hydrogel solution comprising the cells

US 2015/0203820 A1

Jul. 23, 2015

21

can be allowed to harden, for instance in a mold, to form a matrix having cells dispersed therein for implantation. hES-MSC in such a matrix can also be cultured so that the cells are mitotically expanded prior to implantation. The hydrogel is, e.g., an organic polymer (natural or synthetic) that is cross-linked via covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure that entraps water molecules to form a gel. Hydrogel-forming materials include polysaccharides such as alginate and salts thereof, peptides, polyphosphazenes, and polyacrylates, which are crosslinked ionically, or block polymers such as polyethylene oxide-polypropylene glycol block copolymers which are crosslinked by temperature or pH, respectively. In some embodiments, the hydrogel or matrix of the invention is biodegradable. In some embodiments of the invention, the formulation comprises an in situ polymerizable gel (see., e.g., U.S. Patent Application Publication 2002/022676; Anseth et al., J. Control Release, 78(1-3):199-209 (2002); Wang et al., Biomaterials, 24(22):3969-80 (2003).

[0222] In some embodiments, the polymers are at least partially soluble in aqueous solutions, such as water, buffered salt solutions, or aqueous alcohol solutions, that have charged side groups, or a monovalent ionic salt thereof. Examples of polymers having acidic side groups that can be reacted with cations are poly(phosphazenes), poly(acrylic acids), poly(methacrylic acids), copolymers of acrylic acid and methacrylic acid, poly(vinyl acetate), and sulfonated polymers, such as sulfonated polystyrene. Copolymers having acidic side groups formed by reaction of acrylic or methacrylic acid and vinyl ether monomers or polymers can also be used. Examples of acidic groups are carboxylic acid groups, sulfonic acid groups, halogenated (preferably fluorinated) alcohol groups, phenolic OH groups, and acidic OH groups.

[0223] The hES-MSC or co-cultures thereof can be seeded onto a three-dimensional framework or scaffold and implanted in vivo. Such a framework can be implanted in combination with any one or more growth factors, cells, drugs or other components that stimulate tissue formation or otherwise enhance or improve the practice of the invention.

[0224] Examples of scaffolds that can be used in the present invention include nonwoven mats, porous foams, or self-assembling peptides. Nonwoven mats can be formed using fibers comprised of a synthetic absorbable copolymer of glycolic and lactic acids (e.g., PGA/PLA) (VICRYL, Ethicon, Inc., Somerville, N.J.). Foams, composed of, e.g., poly(ϵ -caprolactone)/poly(glycolic acid) (PCL/PGA) copolymer, formed by processes such as freeze-drying, or lyophilization (see, e.g., U.S. Pat. No. 6,355,699), can also be used as scaffolds.

[0225] The hES-MSC can also be seeded onto, or contacted with, a physiologically-acceptable ceramic material including, but not limited to, mono-, di-, tri-, alpha-tri-, beta-tri-, and tetra-calcium phosphate, hydroxyapatite, fluoroapatites, calcium sulfates, calcium fluorides, calcium oxides, calcium carbonates, magnesium calcium phosphates, biologically active glasses such as BIOGLASS®, and mixtures thereof. Porous biocompatible ceramic materials currently commercially available include SURGIBONE® (CanMedica Corp., Canada), ENDOBON® (Merck Biomaterial France, France), CEROS® (Mathys, AG, Bettlach, Switzerland), and mineralized collagen bone grafting products such as HEALOSTM (DePuy, Inc., Raynham, Mass.) and VITOSS®, RHA-

KOSS™, and CORTOSS® (Orthovita, Malvern, Pa.). The framework can be a mixture, blend or composite of natural and/or synthetic materials.

[0226] In another embodiment, hES-MSC can be seeded onto, or contacted with, a felt, which can be, e.g., composed of a multifilament yarn made from a bioabsorbable material such as PGA, PLA, PCL copolymers or blends, or hyaluronic acid.

[0227] The hES-MSC can, in another embodiment, be seeded onto foam scaffolds that may be composite structures. Such foam scaffolds can be molded into a useful shape, such as that of a portion of a specific structure in the body to be repaired, replaced or augmented. In some embodiments, the framework is treated, e.g., with 0.1M acetic acid followed by incubation in polylysine, PBS, and/or collagen, prior to inoculation of the cells of the invention in order to enhance cell attachment. External surfaces of a matrix may be modified to improve the attachment or growth of cells and differentiation of tissue, such as by plasma-coating the matrix, or addition of one or more proteins (e.g., collagens, elastic fibers, reticular fibers), glycoproteins, glycosaminoglycans (e.g., heparin sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratin sulfate, etc.), a cellular matrix, and/or other materials such as, but not limited to, gelatin, alginates, agar, agarose, and plant gums, and the like.

[0228] In some embodiments, the scaffold comprises, or is treated with, materials that render it non-thrombogenic. These treatments and materials may also promote and sustain endothelial growth, migration, and extracellular matrix deposition. Examples of these materials and treatments include but are not limited to natural materials such as basement membrane proteins such as laminin and Type IV collagen, synthetic materials such as EPTFE, and segmented polyurethaneurea silicones, such as PURSPAN™ (The Polymer Technology Group, Inc., Berkeley, Calif.). The scaffold can also comprise anti-thrombotic agents such as heparin; the scaffolds can also be treated to alter the surface charge (e.g., coating with plasma) prior to seeding with stem cells.

5.13 Immortalized hES-MSC

[0229] Mammalian hES-MSC can be conditionally immortalized by transfection with any suitable vector containing a growth-promoting gene, that is, a gene encoding a protein that, under appropriate conditions, promotes growth of the transfected cell, such that the production and/or activity of the growth-promoting protein is regulatable by an external factor. In a preferred embodiment the growth-promoting gene is an oncogene such as, but not limited to, v-myc, N-myc, c-myc, p53, SV40 large T antigen, polyoma large T antigen, E1a adenovirus or E7 protein of human papillomavirus.

[0230] External regulation of the growth-promoting protein can be achieved by placing the growth-promoting gene under the control of an externally-regulatable promoter, e.g., a promoter the activity of which can be controlled by, for example, modifying the temperature of the transfected cells or the composition of the medium in contact with the cells. In one embodiment, a tetracycline (tet)-controlled gene expression system can be employed (see Gossen et al., Proc. Natl. Acad. Sci. USA 89:5547-5551, 1992; Hoshimaru et al., Proc. Natl. Acad. Sci. USA 93:1518-1523, 1996). In the absence of tet, a tet-controlled transactivator (tTA) within this vector strongly activates transcription from ph.sub.CMV*-1, a minimal promoter from human cytomegalovirus fused to tet operator sequences. tTA is a fusion protein of the repressor

US 2015/0203820 A1

Jul. 23, 2015

22

(tetR) of the transposon-10-derived tet resistance operon of *Escherichia coli* and the acidic domain of VP 16 of herpes simplex virus. Low, non-toxic concentrations of tet (e.g., 0.01-1.0 µg/mL) almost completely abolish transactivation by tTA.

[0231] In one embodiment, the vector further contains a gene encoding a selectable marker, e.g., a protein that confers drug resistance. The bacterial neomycin resistance gene (neo^R) is one such marker that may be employed within the present invention. Cells carrying neo^R may be selected by means known to those of ordinary skill in the art, such as the addition of, e.g., 100-200 µg/mL G418 to the growth medium.

[0232] Transfection can be achieved by any of a variety of means known to those of ordinary skill in the art including, but not limited to, retroviral infection. In general, a cell culture may be transfected by incubation with a mixture of conditioned medium collected from the producer cell line for the vector and DMEM/F12 containing N2 supplements. For example, a stem cell culture prepared as described above may be infected after, e.g., five days in vitro by incubation for about 20 hours in one volume of conditioned medium and two volumes of DMEM/F12 containing N2 supplements. Transfected cells carrying a selectable marker may then be selected as described above.

[0233] Following transfection, cultures are passaged onto a surface that permits proliferation, e.g., allows at least 30% of the cells to double in a 24 hour period. Preferably, the substrate is a polyornithine/laminin substrate, consisting of tissue culture plastic coated with polyornithine (10 µg/mL) and/or laminin (10 µg/mL), a polylysine/laminin substrate or a surface treated with fibronectin. Cultures are then fed every 3-4 days with growth medium, which may or may not be supplemented with one or more proliferation-enhancing factors. Proliferation-enhancing factors may be added to the growth medium when cultures are less than 50% confluent.

[0234] The conditionally-immortalized hES-MSC cell lines can be passaged using standard techniques, such as by trypsinization, when 80-95% confluent. Up to approximately the twentieth passage, it is, in some embodiments, beneficial to maintain selection (by, for example, the addition of G418 for cells containing a neomycin resistance gene). Cells may also be frozen in liquid nitrogen for long-term storage.

[0235] Clonal cell lines can be isolated from a conditionally-immortalized human hES-MSC cell line prepared as described above. In general, such clonal cell lines may be isolated using standard techniques, such as by limit dilution or using cloning rings, and expanded. Clonal cell lines may generally be fed and passaged as described above.

[0236] Conditionally-immortalized human hES-MSC cell lines, which may, but need not, be clonal, may generally be induced to differentiate by suppressing the production and/or activity of the growth-promoting protein under culture conditions that facilitate differentiation. For example, if the gene encoding the growth-promoting protein is under the control of an externally-regulatable promoter, the conditions, e.g., temperature or composition of medium, may be modified to suppress transcription of the growth-promoting gene. For the tetracycline-controlled gene expression system discussed above, differentiation can be achieved by the addition of tetracycline to suppress transcription of the growth-promoting gene. In general, 1 µg/mL tetracycline for 4-5 days is sufficient to initiate differentiation. To promote further differentiation, additional agents may be included in the growth medium.

5.14 Assays

[0237] The hES-MSC can be used in assays to determine the influence of culture conditions, environmental factors, molecules (e.g., biomolecules, small inorganic molecules. Etc.) and the like on stem cell proliferation, expansion, and/or differentiation, compared to hES-MSC not exposed to such conditions.

[0238] In a preferred embodiment, the hES-MSC are assayed for changes in proliferation, expansion or differentiation upon contact with a molecule. In one embodiment, for example, the invention provides a method of identifying a compound that modulates the proliferation of a plurality of hES-MSC, comprising contacting said plurality of hES-MSC with said compound under conditions that allow proliferation, wherein if said compound causes a detectable change in proliferation of said hES-MSC compared to a plurality of hES-MSC not contacted with said compound, said compound is identified as a compound that modulates proliferation of hES-MSC. In a specific embodiment, said compound is identified as an inhibitor of proliferation. In another specific embodiment, said compound is identified as an enhancer of proliferation.

[0239] In another embodiment, the invention provides a method of identifying a compound that modulates the expansion of a plurality of hES-MSC, comprising contacting said plurality of hES-MSC with said compound under conditions that allow expansion, wherein if said compound causes a detectable change in expansion of said plurality of hES-MSC compared to a plurality of hES-MSC not contacted with said compound, said compound is identified as a compound that modulates expansion of hES-MSC. In a specific embodiment, said compound is identified as an inhibitor of expansion. In another specific embodiment, said compound is identified as an enhancer of expansion.

[0240] In another embodiment, disclosed herein is a method of identifying a compound that modulates the differentiation of a hES-MSC, comprising contacting said hES-MSC with said compound under conditions that allow differentiation, wherein if said compound causes a detectable change in differentiation of said hES-MSC compared to a hES-MSC not contacted with said compound, said compound is identified as a compound that modulates proliferation of hES-MSC. In a specific embodiment, said compound is identified as an inhibitor of differentiation. In another specific embodiment, said compound is identified as an enhancer of differentiation.

5.15 Therapeutic Uses of Human Embryonic Stem Cell Derived Mesenchymal Stem Cells

[0241] Mesenchymal stem cells derived from bone marrow (BM-MSCs) have been used as cell based therapy for T cell related autoimmune diseases, including multiple sclerosis, but due to limited sources, unstable quality, and biosafety concerns of using cells derived from adult tissue, their use as a therapeutic aid has been limited.

[0242] The novel method for generating mesenchymal stem cells from embryonic stem cells set forth herein, and the novel hES-MSCs generated from this method, provide new therapies for T cell related autoimmune disease, in particular multiple sclerosis.

[0243] In certain embodiments, hES-MSC given to mice pre-onset of EAE, remarkably attenuated the disease score of these animals. The decrease in score was accompanied by

US 2015/0203820 A1

Jul. 23, 2015

23

decreased demyelination, T cell infiltration, and microglial responses in the central nervous system, as well as repressed immune cell proliferation, and differentiation *in vitro*.

[0244] In certain embodiments, a gradual decline of disease score in EAE mice after treatment with hES-MSC, post disease onset, was observed. In certain embodiments, hES-MSC have both prophylactic and therapeutic effects on the disease.

[0245] In certain embodiment, the immunosuppressive activity of the hES-MSC account for the prophylactic effect on the disease as irradiated hES-MSC, which are unlikely to replace damage myelin, were also effective in reducing disease score. In one embodiment, irradiation does not shorten the lifespan of the hES-MSC.

[0246] In certain embodiment, the therapeutic effects of the hES-MSC involve immunosuppression as well as neural repair and regeneration.

[0247] In certain embodiment, EAE mice treated with hES-MSC have much fewer inflammatory T cells in their central nervous system and less T cells infiltrating the spinal cord. The hES-MSC can reduce damage and symptoms caused by inflammatory T cells, making them useful in therapy and prevention of all T cell related autoimmune diseases. hES-MSC also decreased demyelination.

[0248] In certain embodiment, the therapeutic method comprises the use of hES-MSC in combination with other therapeutic agent. In certain embodiment, hES-MSC is administered in combination with anti-IL-6 antibody, anti-IL-12 antibody, and/or other immunosuppressive agents.

[0249] As shown by the results herein, hES-MSCs from three different hES cell lines, given to mice pre-onset of EAE, remarkably attenuated the disease score of these animals. The decrease in score was accompanied by decreased demyelination, T cell infiltration, and microglial responses in the central nervous system, as well as repressed immune cell proliferation, and differentiation *in vitro*.

[0250] Additionally, a gradual decline of disease score in EAE mice after treatment with hES-MSCs, post disease onset, was observed. These data suggest that hES-MSCs have both prophylactic and therapeutic effects on the disease.

[0251] It is believed that the immunosuppressive activity of the hES-MSCs account for the prophylactic effect on the disease as irradiated hES-MSCs, which are unlikely to replace damage myelin, were also effective in reducing disease score. Moreover, irradiation does not shorten the lifespan of the hES-MSCs.

[0252] The therapeutic effect of the hES-MSCs may involve immunosuppression as well as neural repair and regeneration.

[0253] More surprisingly, EAE mice treated with hES-MSCs have much fewer inflammatory T cells in their central nervous system and less T cells infiltrating the spinal cord, showing that the hES-MSCs can reduce damage and symptoms caused by inflammatory T cells, making them useful in therapy and prevention of all T cell related autoimmune diseases. hES-MSCs also decreased demyelination.

[0254] These favorable results are all in marked contrast to the results obtained with bone marrow-derived mesenchymal stem cells. BM-MSCs only suppressed mouse T cell proliferation in response to anti-CD3 stimuli at low doses *in vitro*, and even enhanced Th1 and Th17 cell infiltration into the CNS. Autoreactive effector CD4⁺ T cells have been associated with the pathogenesis of several autoimmune disorders, including multiple sclerosis, Crohn's disease, and rheumatoid arthritis. These CD4⁺ T cells include Th1 and Th17 cells.

[0255] Moreover, BM-MSCs had no effect at all on the score of EAE mice. These observations are surprising but consistent with previous reports that show only mild or negligible effects of human BM-MSCs on EAE mice (Gordon et al. 2008a; Zhang et al. 2005; Payne et al. 2012). A recent report showed a reduction of disease score of only 3.5 to 3.0 of EAE mice treated with human umbilical-derived MSCs (Liu et al. 2012). The results herein and those from these studies highlight the novelty and usefulness of the present invention.

[0256] Additionally, BM-MSC and hES-MSC have very similar global transcriptional profiles, but differentially express some pro- and anti-inflammatory factors. Among them, IL-6 is expressed at a much higher level in BM-MSCs than hES-MSCs. Moreover, IL-6 expression in BM-MSCs was double upon IFN γ stimulation *in vitro*, whereas it remained low in the hES-MSCs.

[0257] IL-6 is pleiotropic cytokine involved in crosstalk between hematopoietic/immune cells and stromal cells, including the onset and resolution of inflammation. IL-6 can promote the differentiation and functions of Th17 cells (Dong, 2008). The levels of IL-6 are elevated in mononuclear cells in blood and in brain tissue from MS patients (Pataneella et al., 2010), as well as in serum in aged humans (Sethe et al., 2006). Mice lacking IL-6 receptor α at the time of T cell priming are resistant to EAE (Leech et al., 2012). Site-specific production of IL-6 in the CNS can re-target and enhance the inflammatory response in EAE (Quintana et al., 2009), whereas IL-6-neutralizing antibody can reduce symptoms in EAE mice (Gijbels et al., 1995). Thus, IL-6 has become a promising therapeutic target for treatment of MS.

[0258] Immunomodulation of peripheral T cell activity and regeneration and repair of neural cells are widely recognized modes of MSC therapeutic action in MS and in EAE (Al Jumah and Abumaree 2012; Auletta et al. 2012; Morando et al. (2012). However, long-term functional neuronal recovery and sustained disease remission in MS needs repair of the damaged BBB and BSCB (Correale and Villa 2007; Minagar et al. 2012). In other words, MS is an inflammatory, neuro-degenerative, and vascular disease, and effective treatment need to target all three component.

[0259] The characteristics of hES-MSCs make them uniquely suited for the treatment of T cell related autoimmune diseases especially multiple sclerosis. In particular, the hES-MSCs can decrease disease scores of EAE mice, but also decrease demyelination and decrease Th1 and Th17 proliferation, and have low expression of IL-6. These latter two characteristics make them suitable to treat other T cell related autoimmune diseases. Additionally, the ability of the hES-MSCs to cross the blood-brain barrier and blood-spinal cord barrier, makes them superior as a treatment and prevention of multiple sclerosis and other autoimmune diseases related to the central nervous system.

[0260] Thus, one embodiment of the present invention is a method of treating or preventing a T cell related autoimmune disease comprising the steps of administering a therapeutically effective amount of solution, cell culture or pharmaceutical preparation comprising human embryonic-mesenchymal stem cells as described in the preceding paragraphs, to the subject in need thereof. The T cell related autoimmune diseases would include but are not limited to multiple sclerosis, inflammatory bowel disease, Crohn's disease, graft versus host disease, systemic lupus erythematosus, and rheumatoid arthritis. The subject is preferably a mammal or avian, and

US 2015/0203820 A1

Jul. 23, 2015

24

most preferably human. The solution, cell culture or pharmaceutical preparation can comprise irradiated or non-irradiated human embryonic-mesenchymal stem cells. The solution, cell culture or pharmaceutical preparation is preferably administered by injection.

[0261] Multiple sclerosis has been categorized into four subtypes: relapsing/remitting; secondary progressive; primary progressive; and progressive relapsing. The relapsing/remitting subtype is characterized by unpredictable relapses followed by long periods of remission. Secondary progressive MS often happens in individuals who start with relapsing/remitting MS and then have a progressive decline with not periods of remission. Primary progressive MS describes a small number of individuals who never have remission after their initial symptoms. Progressive relapsing, the least common subtype, have a steady neurologic decline, and suffer from acute. Thus, further embodiments of the present invention is a method for treating or preventing multiple sclerosis disease in a subject in need thereof, comprising the steps of administering a therapeutically effective amount of solution, cell culture or pharmaceutical preparation comprising human embryonic-mesenchymal stem cells as described in the preceding paragraphs, to the subject in need thereof. The multiple sclerosis can be relapsing/remitting multiple sclerosis, progressive/relapsing multiple sclerosis, primary multiple sclerosis, or secondary multiple sclerosis. The subject is preferably a mammal, and most preferably human. The solution, cell culture or pharmaceutical preparation can comprise irradiated or non-irradiated human embryonic-mesenchymal stem cells. The solution, cell culture or pharmaceutical preparation is preferably administered by injection.

[0262] Multiple sclerosis manifests in a variety of symptom including sensory disturbance of the limbs, optic nerve dysfunction, pyramidal tract dysfunction, bladder dysfunction, bowel dysfunction, sexual dysfunction, ataxia and diplopia attacks.

[0263] A further embodiment of the present invention is a method of treating multiple sclerosis comprising the steps of administering a therapeutically effective amount of solution, cell culture or pharmaceutical preparation comprising human embryonic-mesenchymal stem cells as described in the preceding paragraphs, to the subject in need thereof, wherein there is detectable improvement in at least one of these symptoms, at least two of these symptoms, at least four of these symptoms, at least five of these symptoms or all of these symptoms.

[0264] The Expanded Disability Status Scale (EDSS) is the most commonly used rating scale to evaluate the clinical status of patients with MS. It measures disability along several separate parameters: strength, sensation, brainstem functions (speech and swallowing), coordination, vision, cognition, and bowel/bladder continence. It is a well-accepted measure of disability in MS and it is not particularly difficult or time consuming to perform. The EDSS quantifies disability in eight Functional Systems (FS) and allows neurologists to assign a Functional System Score (FSS) in each of these (Kurtzke 1983).

[0265] Kurtzke defines functional systems as follows:

[0266] pyramidal

[0267] cerebellar

[0268] brainstem

[0269] sensory

[0270] bowel and bladder

[0271] visual

[0272] cerebral

[0273] other

[0274] The EDSS steps 1.0 to 4.5 refer to people with MS who are fully ambulatory. EDSS steps 5.0 to 9.5 are defined by the impairment to ambulation. The clinical meaning of each possible result is the following:

[0275] 0.0: Normal Neurological Exam

[0276] 1.0: No disability, minimal signs on 1 FS

[0277] 1.5: No disability, minimal signs on 2 of 7 FS

[0278] 2.0: Minimal disability in 1 of 7 FS

[0279] 2.5: Minimal disability in 2 FS

[0280] 3.0: Moderate disability in 1 FS; or mild disability in 3-4 FS, though fully ambulatory

[0281] 3.5: Fully ambulatory but with moderate disability in 1 FS and mild disability in 1 or 2 FS; or moderate disability in 2 FS; or mild disability in 5 FS

[0282] 4.0: Fully ambulatory without aid, up and about 12 hrs a day despite relatively severe disability. Able to walk without aid 500 meters

[0283] 4.5: Fully ambulatory without aid, up and about much of day, able to work a full day, may otherwise have some limitations of full activity or require minimal assistance. Relatively severe disability. Able to walk without aid 300 meters

[0284] 5.0: Ambulatory without aid for about 200 meters. Disability impairs full daily activities

[0285] 5.5: Ambulatory for 100 meters, disability precludes full daily activities

[0286] 6.0: Intermittent or unilateral constant assistance (cane, crutch or brace) required to walk 100 meters with or without resting

[0287] 6.5: Constant bilateral support (cane, crutch or braces) required to walk 20 meters without resting

[0288] 7.0: Unable to walk beyond 5 meters even with aid, essentially restricted to wheelchair, wheels self, transfers alone; active in wheelchair about 12 hours a day

[0289] 7.5: Unable to take more than a few steps, restricted to wheelchair, may need aid to transfer, wheels self, but may require motorized chair for full day's activities

[0290] 8.0: Essentially restricted to bed, chair, or wheelchair, but may be out of bed much of day; retains self care functions, generally effective use of arms

[0291] 8.5: Essentially restricted to bed much of day, some effective use of arms, retains some self care functions

[0292] 9.0: Helpless bed patient, can communicate and eat

[0293] 9.5: Unable to communicate effectively or cat/swallow

[0294] 10.0: Death due to MS

[0295] Therefore, a further embodiment of the present invention is a method for treating multiple sclerosis disease in a subject in need thereof, comprising the steps of administering a therapeutically effective amount of solution, cell culture or pharmaceutical preparation comprising human embryonic-mesenchymal stem cells as described in the preceding paragraphs, to the subject in need thereof wherein the subject demonstrates improvement on the Expanded Disability Status Scale of at least one point, and preferably at least two points.

[0296] There are other therapeutic agents that have been used to treat and prevent multiple sclerosis, including but not

US 2015/0203820 A1

Jul. 23, 2015

25

limited to, fingolimod, adrenocorticotrophic hormone (ACTH), methylprednisolone, dexamethasone, IFN β -1a, IFN-1 b, gliatramer acetate, cyclophosphamide, methotrexate, azathioprine, cladribine, cyclosporine, mitoxantrone, and sulfasalazine.

[0297] Therefore, the method of the present invention can further comprise the administration of one or more additional therapeutic agents to the subject, including but not limited to, fingolimod, adrenocorticotrophic hormone (ACTH), methylprednisolone, dexamethasone, IFN β -1a, IFN-1b, gliatramer acetate, cyclophosphamide, methotrexate, azathioprine, cladribine, cyclosporine, mitoxantrone, and sulfasalazine. In a further embodiment, these additional therapeutic agents can be administered prior to, after, or at the same time as the hES-MSCS, or can be conjugated or attached to the hES-MSCS, as described below.

[0298] Other T cell or B cell related autoimmune diseases that can be treated by the disclosed hES-MSC includes, but are not limited to, Alopecia Areata, Anklosing Spondylitis, Antiphospholipid Syndrome, Autoimmune Addison's Disease, Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Autoimmune Inner Ear Disease, Autoimmune Lymphoproliferative Syndrome (ALPS), Autoimmune Thrombocytopenic Purpura (ATP), Behcet's Disease, Bullous Pemphigoid, Cardiomyopathy, Celiac Sprue-Dermatitis, Chronic Fatigue Syndrome, Immune Deficiency Syndrome (CFIDS), Chronic Inflammatory Demyelinating Polyneuropathy, Cicatricial Pemphigoid, Cold Agglutinin Disease, CREST Syndrome, Crohn's Disease, Deigo's Disease, Dermatomyositis, Dermatomyositis-Juvenile, Discoid Lupus, Essential Mixed Cryoglobulinemia, Fibromyalgia-Fibromyositis, Grave's Disease, Guillain-Barre, Hashimoto's Thyroiditis, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia Purpura (ITP), IgA Nephropathy, Insulin Dependent Diabetes (Type I), Type II diabetes, Juvenile Arthritis, Lupus, Meniere's Disease, Mixed connective Tissue Disease, Multiple Sclerosis, Myasthenia Gravis, *Pemphigus Vulgaris*, Pernicious Anemia, Polyarteritis Nodosa, Polychondritis, Polyglanular Syndromes, Polymyalgia Rheumatica, Polymyositis and Dermatomyositis, Primary Agammaglobulinemia, Primary Biliary Cirrhosis, Psoriasis, Raynaud's Phenomenon, Reiter's Syndrome, Rheumatic Fever, Rheumatoid Arthritis, Sarcoidosis, *Scleroderma*, Sjogren's Syndrome, Stiff-Man Syndrome, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Ulcerative Colitis, Uveitis, Vasculitis, Vitiligo, Wegener's Granulomatosis. Or any acute or chronic inflammation related to burning, surgery, injury, and allergy.

5.16 Uses of Human Embryonic Stem Cell Derived Mesenchymal Stem Cells as Delivery Systems

[0299] Because it has been shown that the hES-MSCs of the present invention have the unique ability to cross the blood-brain barrier and the blood-spinal cord barrier, a further embodiment of the present invention is a method of using human embryonic-mesenchymal stem cells for delivery of agents through the blood brain barrier and/or the blood spinal cord barrier, by attaching or conjugating the agent to the human embryonic mesenchymal stem cells to form a complex; and administering the human embryonic mesenchymal stem cells-agent complex to a subject, wherein the human embryonic mesenchymal stem cells cross the blood-brain and/or the blood-spinal cord barrier and deliver the agent to the central nervous system. The human embryonic mesen-

chymal stem cells may be in the form of a single cell, a cell culture, a solution or a pharmaceutical preparation. Agents would include but are not limited to chemicals, drugs, proteins, DNA, RNA, antibodies, and small molecules.

[0300] A further embodiment of the present invention is a delivery system for the delivery of agents through the blood brain barrier and/or the blood spinal cord barrier comprising human embryonic-mesenchymal stem cells and an agent conjugated or attached to the human embryonic-mesenchymal stem cells.

[0301] The ability to permeate the blood-brain barrier and the blood-spinal cord barrier would be useful in the treatment and prevention of diseases including but not limited to neurological disorders, multiple sclerosis, cancer, Parkinson's Disease, Alzheimer's Disease, Huntington's Disease, meningitis, encephalitis, rabies, epilepsy, dementia, Lyme's Disease, stroke, and amyotrophic lateral sclerosis, as well as brain and spinal cord injury. Thus, a subject in need thereof would have a disease or be at risk for a disease in which the blood-brain barrier and/or blood-spinal cord barrier is involved. Thus, a further embodiment of the present invention is a method of treating a disease or injury, by attaching or conjugating an agent to the human embryonic mesenchymal stem cells to form a complex, and administering the human embryonic mesenchymal stem cells-agent complex to a subject in need thereof, wherein the human embryonic mesenchymal stem cells cross the blood-brain and/or the blood-spinal cord barrier and deliver the agent to the central nervous system, and the agent is used as a treatment or prevention of the disease or injury of the subject. Since the hES-MSC have strong ability migration and infiltration ability, it can also been used as carrier for tumor/cancer therapy to carry anti-tumor drugs and proteins. The human embryonic mesenchymal stem cells may be in the form of a single cell, a cell culture, a solution or a pharmaceutical preparation. Agents include, but are not limited to, chemicals, drugs, proteins, DNA, RNA, micro-RNA, non-coding RNA, antibodies, small molecules and/or nano particles.

[0302] Agents that are useful in the treatment and prevention of diseases include, but not limited to, antibiotics, antiviral agents, anti-fungal agents, steroids, chemotherapeutics, anti-inflammatories, cytokines, and/or synthetic peptides.

[0303] Proteins and peptides would also be useful to conjugate to the hES-MSCs and would include erythropoietin (EPO), anti-beta-amyloid peptides, tissue plasminogen activator (TPA), granulocyte colony stimulating factor (G-CSF), interferon (IFN), growth factor/hormone, anti-VEGF peptides, anti-TNF peptides, NGF, HGF, IL-2, CX3CL1, GCV, CPT-11, cytosine deaminase, HSV-TK, carboxyesterase, oncolytic virus, TSP-1, TRAIL, FASL, IL-10, TGF β . Proteins and peptides that bind to particular receptors and block these receptors would also be useful and are contemplated by the current invention to be attached to the hES-MSCs.

[0304] DNA and RNA that coded for therapeutic proteins and peptide would also be useful to conjugate to the hES-MSCs for delivery across the blood-brain barrier and/or the blood-spinal cord barrier.

[0305] The terms "antibody" and "antibodies" include polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, single chain Fv antibody fragments, Fab fragments, and F(ab')₂ fragments. Polyclonal antibodies are heterogeneous populations of antibody molecules that are specific for a particular antigen, while monoclonal antibodies are homogeneous populations of antibodies to a particular

US 2015/0203820 A1

Jul. 23, 2015

26

epitope contained within an antigen. Monoclonal antibodies are particularly useful in the present invention.

[0306] Any agent that would block the activation, expression and/or action of a molecule or the receptor of the molecule in the pathway related to any disease in which crossing the blood-brain barrier and/or blood-spinal cord barrier is useful could be attached or conjugated to the hES-MSCs. Such agents include but are not limited to chemicals, phytochemicals, pharmaceuticals, biologics, small organic molecules, antibodies, nucleic acids, peptides, and proteins.

[0307] Inhibiting a pathway can also be effected using “decoy” molecules which mimic the region of a target molecule in the pathway binds and activates. The activating molecule would bind to the decoy instead of the target, and activation could not occur.

[0308] Inhibition can also be effected by the use of a “dominantly interfering” molecule, or one in which the binding portion of activating molecule is retained but the molecule is truncated so that the activating domain is lacking. These molecules would bind to receptors in the pathway but be unproductive and block the receptors from binding to the activating molecule. Such decoy molecules and dominantly interfering molecule can be manufactured by methods known in the art, and attached or conjugated to the hES-MSCs for delivery across the blood-brain or blood-spinal cord barrier.

[0309] A method for delivery of agents across the blood-brain and/or blood-spinal cord barrier is also useful for diagnostic agents, including but not limited to chemicals, antibodies, peptides, proteins, DNA, and RNA. Such agents in order to be useful for diagnosis must have a means of being visualized and/or quantified. Such means include, but are not limited to, fluorescence, biomarkers, dyes, radioactive isotopes labels and/or nanoparticles.

[0310] Such a method for delivery and a delivery system would be useful for the diagnosis neurological disorders, multiple sclerosis, cancer, Parkinson’s Disease, Alzheimer’s Disease, Huntington’s Disease, meningitis, encephalitis, rabies, epilepsy, dementia, Lyme’s Disease, stroke, and amyotrophic lateral sclerosis, as well as brain and spinal cord injury. Thus, a further embodiment of the present invention is a method of diagnosing a disease or injury, by attaching or conjugating the agent to the human embryonic mesenchymal stem cells to form a complex, and administering the human embryonic mesenchymal stem cells-agent complex to a subject in which a disease is suspected, wherein the human embryonic mesenchymal stem cells cross the blood-brain and/or the blood-spinal cord barrier and deliver the agent to the central nervous system. The human embryonic-mesenchymal stem cells may be in the form of a single cell, a cell culture, a solution or a pharmaceutical preparation. Agents would include but are not limited to chemicals, drugs, proteins, DNA, RNA, antibodies, and small molecules.

[0311] Agents, no matter the type and whether for treatment, prevention, or diagnosis, can be conjugated or attached to the hES-MSCs by any method known in the art including but not limited to synthetic extracellular matrix, alginate-poly-L-Lysine encapsulate and/or container.

[0312] In certain embodiments, large scale production at industrial level of manufacturing is included in the present disclosure, methods of which are well known in the art. In certain embodiment, the large scale production includes the use of Hyper-STACK 2D culture system and/or Microcarrier 3D bioreactor.

6. EXAMPLES

[0313] The present invention may be better understood by reference to the following non-limiting examples, which are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed to limit the broad scope of the invention.

6.1 Example 1

Derivation of hES-HB-MSCs

[0314] Using a method to derive HB from hESC via EB, mesodermal cells (MP) were enriched and further differentiated into either HB or MSC depending on subsequent culture conditions.

Material and Methods

[0315] Four hESC cell lines were used: H9 (derived from WiCell Research Institute) (Thomson et al. (1998)); CT2 (derived from University of Connecticut Stem Cell Core (Lin et al. (2010)); MA09 (an FDA approved, clinical-grade cell line derived at Advanced Cell Technology, Inc.) (Klimanskaya et al. (2006)); and ES03-Envy (Envy, a GFP-labeled line, derived at ES International) (Costa et al. (2005)). These cell lines were cultured on Matrigel (BD Biosciences, San Jose, Calif.) and cultured in TeSR1 medium, (Stem Cell Technologies, Vancouver, Canada), with or without adding of 0.05-0.2 μ M of BIO (6-Bromindirubin-3'-oxime (CAS 667463-62-9)).

[0316] hESC cells were then differentiated into EB cells and then enriched for HB as previously described (Lu et al. (2008); Lu et al. (2007)). 50-80% confluent hEs cell on the Matrigel plate were digested with Dispase (1 mg/ml for 5 to 10 minutes) and then washed with EB formation basal medium, HPGM (Lonza, Walkersville, Md.), or STEMLINE I/II Hematopoietic Stem Cell Expansion Medium (Sigma, St. Louis, Mo.), or StemSpan H3000 (Stem Cell Technologies, Vancouver, Canada), or IMDM with 10% FBS, or DMEDM/F12 with 10% FBS. Cells were then cultured in EB formation medium supplemented with 50 ng/ml of VEGF (Peprotech) and 50 ng/ml of BMP4 (Stemgent) for 48 hours on ultra-low plate at a density of about 2-3 million cells/ml. After 48 hours, half the culture medium was replaced with fresh EB formation medium plus 25-50 ng/ml of bFGF.

[0317] Four days later, EB cells formed in the medium were harvested and dissociated into single cells with TrypLE (Invitrogen) at 37° C. for 2-3 minutes. Cells were washed and resuspended at 1-5 million cells/ml in EB formation basal medium. The single cell suspension were then mixed at 1:10 with Hemangioblast Growth Medium (Stem Cell Technologies, Vancouver, Canada).

[0318] Blast cell growth medium (BGM) were made as follows: To 100 ml Serum-free methylcellulose CFU medium (Stem Cell Technologies, H4436 or H4536), added with VEGF, TPO and FLT3-Ligand to 50 ng/ml, bFGF to 20-50 ng/ml, 1 ml of EX-CYTE Growth Enhancement Media Supplement and 1 ml of Pen/Strap, mix well.

[0319] The mixtures were vortexed and plated onto ultralow plates by passing through a 16 G needle and cultured for 5-9 days at 37° C. with 5% CO₂.

[0320] Single cells were then re-suspended in MSC medium containing: 1) 10-20% FBS in alpha-MEM (Invitrogen) or 2) 10-20% KOSR alpha-MEM, 3) 10-20% FBS DMEM high-glucose, or 4) 10-20% KOSR DMEM high-

US 2015/0203820 A1

Jul. 23, 2015

27

glucose, and cultured on either Matrigel, gelatin, vitronectin, fibronectin, collagen I coated plates at a density of 100-5,000 cell/cm². The medium was changed after 24 hours and refreshed every 2-4 days. After 6-12 days the cells gradually differentiated into spindle-like cells similar to typical MSCs.

[0321] Flow cytometry staining was used to characterize the hES-MSCs. Cells were washed and blocked with 2% BSA in PBS, and stained with antibodies for various cell surface markers CD31, CD34, CD29, CD73, CD90, CD105, CD44, CD45, CD146, CD166, HLA-ABC, HLA-DR, HLA-G (BD Bioscience or eBioscience) by following the manufacturers' instructions. Data were collected on FACS LSR II Flow Cytometer using FACSDiva software (BD Bioscience). Post-acquisition analysis was performed with the FlowJo software (Treestar).

Results

[0322] On day 9 of the culture (HB-d9), characterization by flow cytometry showed 68.1% CD45+ cells (hematopoietic progenitors), 22.1% CD31+ cells (epithelial progenitors), and 9.7% CD+ 73 cells (MSC). (FIG. 2)

[0323] These cells were replated onto Matrigel-coated plates containing MSC growth medium (Invitrogen). Twenty-four (24) hours later 5-10% of the cells attached to the plate and 9-14 days later, the attached cells fully differentiated into MSC-like cells.

[0324] We have also found that by adding GSK3 inhibitor BIO in the feeder free serum free hESC culture can significantly increase the EB and HB formation efficiency. As shown in FIG. 28, adding BIO in the mTesr1 medium can increase the size and yield of the EB culture afterwards. As shown in FIG. 29, total EB number was increase 3 folds after using BIO in the mTesr1 Medium. As shown in FIG. 30, the percentage of CD45 cell differentiation which is an indicator of the hemangioblast differentiation efficiency is also tripled with BIO treatment compare to traditional mTesr1 Medium.

[0325] We also found that after differentiation, CD10 expression level varies between different lines of hESC lines. As shown in FIG. 31, hES-MSC from MA09 have extreme high level of CD10, but hES-MSC from H9 and CT2 has lower CD10 expression similar to that from BM-MSC. This is confirmed by both microarray and FACS staining.

6.2 Example 2

Further Characterization of hES-HB-MSC Cells

[0326] The MSC cells obtained in Example 1 were further analyzed using flow cytometry, immunofluorescence staining, multi-lineage differentiation, and karyotyping.

Materials and Methods

[0327] Flow cytometry was performed as described in Example 1.

[0328] Immunofluorescence was performed by fixing cells with 4% paraformaldehyde for 15 minutes, and incubating in PBS containing 0.2% Triton X-100 (for permeabilization) and 5% goat serum (for blocking). PBS containing 5% goat serum was used to dilute the primary antibodies. The cells were incubated with the primary antibodies at 4° C. overnight, followed by washing with PBS for three times. Afterwards, the cells were incubated with fluorochrome-conjugated, corresponding secondary antibodies at room temperature for 30 minutes and washed with PBS for three

times. Finally, the cells were examined under fluorescence microscope to capture both phase and fluorescent images.

[0329] The G-banded karyotyping of hES-MSC was conducted through an outsourced service at University of Connecticut-Storrs Laboratory.

[0330] Microarray Analysis:

[0331] HumanHT-12 V3 expression BeadChip (illumina) was used for microarray, genomic studio V2011.1 was used for data analysis.

Results

[0332] The attached cells obtained 9-14 days later, fully differentiated into MSC-like cells with cell surface markers similar to those of BM-MSCs. The hES-MSCs expressed high levels of CD73 (greater than 99%), CD90 (greater than 90%), CD105 (greater than 90%), CD13 (greater than 85%), CD29 (greater than 90%), CD54 (greater than 80%), CD44 (greater than 99%), and CD166 (greater than 90%), but did not express non-MSC markers such as CD31, CD34 and CD45 (FIGS. 2 and 3).

[0333] The hES-MSCs could be cultured in vitro for up to 10 passages with normal karyotyping (FIG. 4) and sustained expressional profiles of their cell surface markers and differentiation capability.

6.3 Example 3

hES-MSCs Attenuate the Disease Score of EAE Mice in Both Prophylactic and Therapeutic Modes

[0334] Because it has been shown that BM-MSCs can attenuate the disease progression of the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), the hES-MSCs obtained in Example 1 were injected into mice with EAE to determine if they would have the same effect.

Materials and Methods

[0335] The mouse EAE model was induced as previously described (Stromnes and Goverman (2006)). C57BL/6 mice were subcutaneously injected with a mixture of myelin oligodendrocyte glycoprotein peptide 35-55 (MOG⁸⁸⁻⁸⁸), Freund's adjuvant, and pertussis toxin contained in the EAE Induction Kit (Hooke Laboratories, Inc, Mass. (Cat. #EK-0114)) following the manufacturer's protocol and as described in Ge et al. (2012).

[0336] BM- or hES-MSC at 10⁶ cells/mouse or PBS (a vehicle control) was intraperitoneal (i.p.) injected on day 6 (for pre-onset) or 18 (for post-onset) after the immunization. The disease score was monitored on the mice every day for up to 31 days.

[0337] The disease scoring system is as follows:

[0338] 0: no sign of disease;

[0339] 1: loss of tone in the tail;

[0340] 2: partial hind limb paralysis;

[0341] 3: complete hind limb paralysis;

[0342] 4: front limb paralysis; and

[0343] 5: moribund

(Stromnes and Goverman, 2006).

Results

[0344] As shown in FIG. 5, the hES-MSCs derived from the three hESC lines CT2, MA09, and H9 all significantly attenu-

US 2015/0203820 A1

Jul. 23, 2015

28

ated the daily disease scores, as well as the cumulative and maximal disease scores (FIG. 6) when injected at 6 days or pre-onset of disease, showing a prophylactic effect of the hES-MSCs. Mice injected with CT2 hESCs manifested high disease scores similar to those seen with control mice receiving PBS injection, ruling out the possibility of the effect of human xenograft in mice.

[0345] As shown in FIG. 7, treatment with hES-MSCs also had a therapeutic effect on mice that have already developed EAE. When injected with hES-MSCs on day 18 post-immunization (right after the disease score peaked in mice), there was a gradual decline of the disease score in hES-MSC-treated EAE mice with an average score of 1.67 at day 30, whereas the PBS-treated EAE mice had an average score of 2.8 at the same day.

6.4 Example 4

Characterization of the Central Nervous System of the EAE Mice Treated with hES-MSCs

[0346] The central nervous system of the EAE mice treated with hES-MSCs was further analyzed.

Materials and Methods

[0347] Flow cytometry as described in Example 1 was used. Regulatory T cells in the CNS of EAE mice treated with PBS or hES-MSC (CT2) as described in Example 3 were analyzed day 15 post-immunization through FACS analysis of Foxp3 and CD25.

[0348] Th1 and Th17 cells from the CNS of EAE mice treated with PBS or hES-MSC (CT2) as described in Example 3 were analyzed day 15 post-immunization by perfusing EAE mice with 20 ml cold PBS through the left ventricle. The brain and spinal cord were harvested from the perfused mice and ground into small pieces. After digestion with collagenase (1 mg/ml) and Dispase (1 mg/ml) for 20 minutes at 37°C., the tissues were further ground and passed through a 40-µm strainer. Cells were washed and re-suspended in 4 ml of 40% Percoll and overlaid onto 5 ml of 70% Percoll. After centrifugation at 2,000 rpm for 20 minutes cells in the inter layer were collected. The cells were then stimulated with 12-O tetradecanoylphorbol-13-acetate (TPA) at 50 ng/ml (Sigma, Mo.) and ionomycin at 500 ng/ml (Sigma, Mo.) in the presence of GolgiStop (BD Bioscience, Calif.) for 6 hours. Cells were then immunostained with anti-CD4-FITC and anti-CD8-Pacific Blue antibodies (BD Bioscience, Calif.). For intracellular staining of IFNγ and IL-17A, cells were fixed and permeabilized using an intracellular staining kit (BD Bioscience, Calif.) by following the manufacturer's instructions.

Pathology of the Spinal Cord

[0349] Paraffin-embedded spinal cord cross sections were immunostained for CD3 (Biocare Medical) or Iba1 (Wako) and counterstained with anti-MBP (Millipore) or fluoromyelin as previously described (Crocker et al., 2006; Moore et al., 2011). Quantification of anti-MBP staining intensity was performed on the lateral columns of sections from the thoracic and lumbar levels of spinal cord samples using ImageJ (NIH) (Crocker et al., 2006). At least three regions of interest were analyzed for each subject in each treatment group.

Results

[0350] Because of the important role of immune-suppressive regulatory T cells (T_{reg} cells detected as Foxp3- and CD25+) in suppressing inflammation (Hansen et al., (2008)), the ratio of T_{reg} cells among T cells infiltrated into the CNS of EAE mice treated with hES-MSCs and PBS was looked at, and it was found that the ratio did not increase (FIG. 8), suggesting that T_{reg} cells may not be responsible for the immunosuppressive effect of hES-MSCs. This is similar to the case with BM-MSC on EAE mice reported by others (Zappia et al. (2005)).

[0351] Much fewer CD4+, CD8+, Th1, and Th17 T cells were isolated from the CNS in the hES-MSC treat group than in the control PBS or hESC-injected group (FIG. 9), suggesting that reduction of these inflammatory T cells may contribute to the improvement of the symptoms of the hES-MSC-treated mice.

[0352] Pathologically, the microglial response in the spinal cord was analyzed which is a sign of inflammatory responses in the CNS, via immunostaining for ionized calcium-binding adapter molecule 1 (IBA1). IBA1 staining was diminished in the spinal cord of EAE mice treated with hES-MSC compared to those treated with PBS (FIG. 10). Consistently, infiltration of total T cells (stained as CD3+ cells) into the spinal cord was decreased in the hES-MSC-treated EAE mice compared to the control (FIG. 10).

[0353] Immunostaining for myelin-binding protein (MBP) in the spinal cord indicates that demyelination was reduced in the mice treated with hES-MSC compared to the mice treated with PBS (FIGS. 10 and 11), suggesting that hES-MSC can prevent and/or repair the inflammatory damages in the CNS and preserve the integrity of myelin.

6.5 Example 5

hES-MSCs have a Stronger Anti-EAE Effect In Vivo than BM-MSC with Evidence in the CNS

[0354] BM-MSC have long been used to treat autoimmune diseases including MS on various animal models and clinical trials, however the outcomes include mixed or improved response or no change (Tyndall, 2011). Thus, hES-MSCs were compared with human BM-MSC in this study.

Materials and Methods

[0355] EAE mice as described in Example 3 were i.p. injected at 6 days post-immunization with PBS, BM-MSC or hES-MSCs (MA09) (10^6 cells from three different sources). Disease scoring was done as described in Example 3.

[0356] Flow cytometry of T cells infiltrated into the CNS of the BM-MSC-treated mice was performed as described in Example 4.

Results

[0357] Five of human BM-MSC line obtained from six sources failed to attenuate the disease score of EAE mice, only one BM-MSC line mildly decreased disease score by 1, in marked contrast to hES-MSC that again showed strong anti-EAE effect (FIG. 12).

[0358] T cells infiltrated in the CNS of the EAE mice treated with BM-MSC were found to have higher ratio of Th17 cells among the CD4 T cells in the CNS than both the PBS- and hES-MS-treated mice, suggesting that BM-MSC can increase the differentiation of Th17 cells. After calculat-

US 2015/0203820 A1

Jul. 23, 2015

29

ing the total infiltrated cell Th1 and Th17 cells, it was found that all 3 lines of BM-MSC significantly increased the total Th17 cell number (FIG. 13), and only one line can increase the total Th1 cell number, whereas hES-MSCs can decrease both Th1 and Th17 as shown in Example 4. Together, this indicates that BMMSC not only failed to inhibit the Th1 response, rather they increased the Th17 response in vivo. Reduced fluoromyelin staining of MBP in the spinal cord confirms severe damage in both PBS-treated and BM-MSC-treated mice while MBP levels were preserved in hES-MSC-treated mice (FIG. 14). The damaged regions in BM-MSC-treated mice also show a high number of DAPI positive cells, suggesting more inflammatory cells infiltration.

6.6 Example 6

hES-MSCs can Enter the Central Nervous System

[0359] Since it is well known that the BBB and BSCB play a key role in preventing T cells from entry into the CNS, it was asked whether they also play a role in the entry of MSC into the CNS.

Materials and Methods

[0360] MSC differentiated from the hESC line Envy that constitutively expressed GFP (Costa et al., 2005) as described in Example 1, and GFP-labeled human BM-MSC (Hofstetter et al., 2002), were injected them into EAE mice 6 days post-immunization, and isolated the spinal cord 14 days post-immunization.

[0361] The migration of the GFP+ cells through the BSCB in the spinal cord

Results

[0362] As shown in FIG. 18, only the GFP+ hES-MSC migrated out of the vessels and aggregated in the parenchyma of the perivascular regions of the spinal cord, however, the GFP+ BM-MSC were observed only inside the vessels. Parenchymal inflamed venules showed "parenchymal localization" of GFP+hES-MSC, indicating they have extravasated. In contrast, the mice with GFP+BM-MSC appeared to have more "lumen restricted" GFP immunoreactivity (FIG. 15A). Inflamed meningeal venules also displayed limited extravasation of GFP+BM-MSCs when compared to mice receiving GFP+hES-MSCs (FIG. 15B).

[0363] These results indicate that only hES-MSC, and not BM-MSC, have the requisite mechanism to exit the vessels efficiently to fulfill enter into the CNS.

6.7 Example 6

hES-MSCs have a Stronger Inhibition on T Cell Functions In Vitro than BM-MSC

[0364] hES-MSCs and BM-MSCs were compared for their ability to inhibit T cell proliferation in vitro following antigen stimulation.

Materials and Methods

[0365] The in vitro assay for T cell proliferation was performed using lymphocytes isolated from mouse peripheral lymph nodes. These lymphocytes were labeled with 5 μ M of and labeled the cells with carboxyfluorescein succinimidyl ester (CFSE) to track their proliferation by monitoring CFSE

dilution in their daughter cells, for 10 minutes at 37° C. 10,000 hES-MSCs or BM-MSCs were mixed with 100,000 lymphocytes per well in a 96-well plate, and the cells were stimulated for proliferation with plate-bound anti-CD3 (at 0.2, 0.6, 2, and 6 μ g/ml) and soluble anti-CD28 antibodies (eBioscience, Calif.). The cells were collected 3 days after the stimulation, followed by FACS staining with anti-CD4 and anti-CD8 antibodies (BD Bioscience, Calif.). CFSE dilution was gated on CD4 and CD8. T cells, respectively.

Results

[0366] Using the in vitro assay with mouse lymphocytes, it was found hES-MSCs inhibited the proliferation of mouse CD4+ and CD8+ T cells when stimulated with anti-CD3 antibody at 0.2 and 0.6 μ g/ml, or 0.1 and 0.3 μ g/ml, whereas BM-MSC only did so when the T cells were stimulated with anti-CD3 antibody at low doses, i.e., 0.2 or 0.1 μ g/ml (FIG. 16, 17)

6.8 Example 7

Further Evidence that hES-MSCs have a Stronger Inhibition on T Cell Functions In Vitro than BM-MSC

Materials and Methods

[0367] In vitro assays of Th0, Th1, and Th17 cells were performed using naive mouse CD4+ T cells isolated from mouse spleen and purified with the Naive CD4 T Cell Enrichment kit (Stem Cell Technologies, Canada). The cells were incubated with hES-MSCs or BM-MSCs at a ratio of 1:10 or PBS, followed by Th1 or Th17 differentiation for 5 days. Cells activated with anti-CD3 and anti-CD28 antibodies under the following conditions. Cells were cultured with rhIL-2 at 5 ng/ml to remain in Th0 status, with anti-mIL-4 at 5 μ g/ml (eBioscience, Calif.), rhIL-2 at 5 ng/ml (eBioscience, Calif.), and mIL-12 at 10 ng/ml (PeproTech, Calif.) for Th1 differentiation, and with anti-mIL-4 at 5 μ g/ml, anti-mIFN γ at 5 μ g/ml (eBioscience, Calif.), rmIL-6 at 20 ng/ml (PeproTech, Calif.), and rhTGF- β 1 at 1 ng/ml (PeproTech, Calif.) for Th17 differentiation. For some groups, anti-hIL-6 antibody (eBioscience, Calif.) was added to culture at 10 μ g/ml. Naive CD4 T cells were plated at 0.3×10^6 cells/well in 24-well plates. hES- or BM-MSC that had been mitotically-arrested through irradiation at 80 Gy, were added 1 hour after. IFN γ + and IL-17+ cells were determined via FACS.

Results

[0368] Since EAE mice treated with BM-MSC had more Th17 and Th1 cell infiltration into the CNS than mice treated with hES-MSC as shown in Example 5 and FIG. 18, these T cell subtypes were examined in vitro in the presence or absence of hES- and BM-MSC.

[0369] As shown in FIG. 18, under the Th1 condition, differentiation into Th1 (IFN γ +/-IL-17-) cells was reduced by hES-MSC (from 29.7% to 18.0%) In comparison, all BM-MSC lines either did not change or increased the Th1 differentiation (26.9% to 43.6%).

[0370] Under the Th17 differentiation condition, both hES- and BM-MSC reduced the ratio of Th17 cells, but surprisingly all BM-MSC lines significantly increased the percentage of IFN γ producing cells but not hES-MSCs.

US 2015/0203820 A1

Jul. 23, 2015

30

[0371] These results suggest that BM-MSc cannot inhibit Th1 differentiation under the Th1 condition, and increased Th1 cell differentiation under Th17 conditions. These complex effects of BM-MSc in vitro may mirror the mixed effects on EAE mice.

6.9 Example 8

Gene Expression Profiles of hES-MSCs and BM-MSCs

[0372] Microarray analysis was performed to compare the gene expression profile of hES-MSCs and BM-MSCs.

Materials and Methods

[0373] For microarray analysis RNA of hES-MSc at passages 2-4 or BM-MSc at passage 3 were harvested with Trizol (Invitrogen, Calif.) following manufacturer's protocol. The HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, Calif.) was used to analyze the gene expression profile of the cells. Data were analyzed using Genome Studio V2011.1. Two BM-MSc cell lines from different sources were used, and two hES-MSc cell lines, derived from H9 and MA09, were used.

[0374] Flow cytometry analysis was performed as in Example 1.

Results

[0375] As shown in Table 1, the overall expressional profiles of the hES- and BM-MSc samples were quite similar with most of the anti-inflammatory genes such as HLA-G, HGF, COX2, IDO1, and iNOS expressed at similar levels among these samples.

TABLE 1

	hES-MSc		BM-MSc	
HLA-G	359.1	±52.2	726.8	±51.3
HGF	116.9	±19.1	115	±15.3
PTGS1/COX-1	136.3	±2.5	122.7	±14.1
PTGS2/COX-2	346.2	±244.5	964	±674.7
IDO1	121.9	±3.9	127.6	±12.4
IDO2	119.9	±1.8	123.5	±5.46
NOS2/iNOS	129.5	±10.6	154.2	±11.2

[0376] However, a small set of genes was expressed differentially between the hES-MSCs and BM-MSCs samples. These genes include those encoding anti-inflammatory factors, such as IL-10 and TGFβ and pro-inflammatory factors, such as, CCL2, MMP2, RAGE, IFNγR1, IFNγR2, IL-12, IL-6, and VCAM1 (FIG. 19).

[0377] The higher expression of IL-6 in BM-MSc than hES-MSc was confirmed through intracellular staining followed by flow cytometry analysis as shown in FIG. 20.

[0378] Based on microarray analysis, the transcriptomic profiles of hES-MSc and BM-MSc are quite similar. Only a small set of genes has differential expression between hES-MSc and BM-MSc. These genes include those encoding potential anti-inflammatory factors (AIFs) and pro-inflammatory factors (PIFs) (FIGS. 35 and 36). Among the differentially expressed factors in hES-MSc and BM-MSc, some potential AIFs such as IL-10 (Dai et al., 2012) and TGFβ2 (Huss et al., 2011) are expressed higher in hES-MSc than BM-MSc, whereas some potential PIFs such as IL-6 (Quintana et al., 2009), IL-12 (Becher et al., 2002), CCL2 (Mahad

and Ransohoff, 2003), VCAM1 (Chaudhary et al., 2006), RAGE (Cuccurullo et al., 2006), and MMP2 (Cunnea et al., 2010) have lower expression in hES-MSc than BM-MSc (FIGS. 39A-B). The lower ratio of IL-6+ cells among hES-MSc than BM-MSc was confirmed via FACS (FIGS. 37A-D).

[0379] Provided herein is a method of identification of highly immunosuppressive hES-MSc. The method comprises using expression level of IL-6 and other factors mentioned above as indicator of the quality of hES-MSc and BM-MSc. Provided herein are hES-MSc that has <5% of IL-6 positive cells, and low level of IL12, TNFα, RAGE, and other pro-inflammatory cytokines. Also preferred are hES-MSc that express high level of TGFβ and IL-10. Provided herein are methods of producing hES-MSc that has improved immunosuppressive function by genetic and epigenetic modification of differentially expressed factors.

6.10 Example 9

BM-MSCs, but not hES-MSCs, Produce IL-6 in Response to Activated T Cells

[0380] Since it is known that MSCs can be activated to produce cytokines including IL-6 following stimulation with factors such as IFNγ (Ryan et al., 2007), TNFα (English et al., 2007), Toll-like receptor, CCL2, CCL5, and IL-8 in vitro and in vivo (Anton et al., 2012; Waterman et al., 2010), it is possible that MSC may produce more IL-6 in contact with activated T cells as the latter produces high levels of IFNγ and TNFα.

Materials and Methods

[0381] BM-MSCs and hES-MSCs (MA09) were co-cultured with human PBMC for four days. 2.5 ng/ml of PHA was added for the final two days to stimulate T cells. The PBMC was washed away and quantitative RT-PCR was performed as described in Example 8.

[0382] MSCs were stimulated with directly with IFNγ in vitro at 10 ng/ml for 12 hours, and IL-6 was detected via intracellular FACS.

Results

[0383] When the MSC were directly stimulated with IFNγ in vitro, it was found that IL-6 expression in hES-MSc did not change following the stimulation, but IL-6 level was almost doubled in IFNγ-treated BM-MSc compared to the already high, basal level in the untreated BM-MSc (FIG. 21). These results suggest that BM-MSc, but not hES-MSc, produce high levels of the proinflammatory factor IL-6 in response to activated T cells or inflammatory cytokines, probably another reason for the compromised immunosuppressive effect of BM-MSc.

6.11 Example 10

Further Evidence of IL-6 Involvement in BM-MSc Limited Ant-EAE Activity

[0384] Since IL-6 enhances T cell proliferation and survival, and promote T17 cell differentiation (Dienz and Rincon, 2009; Rochman et al., 2005), elevated IL-6 production in BM-MSc may counteract the otherwise anti-inflammatory

US 2015/0203820 A1

Jul. 23, 2015

31

activity of BM-MSC. To test this possibility, we used a neutralizing anti-human IL-6 antibody in the following experiments.

Materials and Methods

[0385] The in vitro assay for T cell proliferation described in Example 6 was used. CFSE labeled mouse lymphocytes were incubated with and without BM-MSCs at a ratio of 1:10 and anti-human IL-6 antibody at 10 µg/ml. Ratios of divided CD4+ and CD8+ T cell was determined CFSE FACS staining.

[0386] hES-MSCs or BM-MSCs were incubated with mouse naïve CD4+ T cells at a ratio of 1:10 under the Th17 differentiation conditions for five days as described in Example 7 in the presence or absence of 10 g/ml of anti-human IL-6 antibody. IFNγ+ and IL-17+ cells were determined via intracellular FACS staining after TPA/ionomycin stimulation.

Results

[0387] As shown in FIG. 22, 23, the anti-IL-6 antibody enhanced the suppressive effect of different BM-MSC lines on mouse CD8+ T cell (FIG. 22, 23B) and CD4+ T cell (FIG. 23A) proliferation in response to anti-CD3 antibody stimulation.

[0388] Next, it was examined whether adding anti-human-IL-6 antibody can reverse the increase of Th1 differentiation in vitro. Under a special differentiation condition, so called Th0 condition, no mouse cytokine was added but anti-mouse-IFNγ and anti-mouse-IL-4 was used to inhibit both Th1 and Th2 differentiation. Co-cultured mouse T cells with human BM-MSC but not hES-MSC can dramatically increase the Th1 differentiation of mouse T cells. Using anti-human-IL-6 antibody can reduce this effect by 30%-50% (FIG. 24). Thus, the elevated IL-6 production in BM-MSC may be at least partially responsible for the compromised anti-inflammatory and anti-EAE effects of the cells, in sharp contrast to hES-MSC which have low IL-6 production.

6.12 Example 11

Irradiated hES-MSCs Retain Anti-EAE Effect

[0389] Mouse embryonic fibroblasts (MEF) are routinely irradiated to stop mitosis without affecting their feeder activity to sustain self-renewal and pluripotency of hESC, as used since the first derivation of hESC lines (Thomson et al. (1998)). Based upon this, it was hypothesized that irradiated MSC may also sustain the anti-EAE effect exerted by non-irradiated MSC.

Materials and Methods

[0390] hES-MSC, derived from MA09 hESC were irradiated at 80 Gy right before injecting them into EAE mice at 10⁶ cells/mouse at day 6 post-immunization as described in Example 2. Disease scoring was done as described in Example 2.

Results

[0391] As shown in FIG. 25, a decrease of the disease score in the injected mice was found although milder than the decrease caused by non-irradiated hES-MSC. When the dose

of hES-MSC was increased to 2×10⁶ cells/mouse, similar anti-EAE effects were seen between the irradiated and non-irradiated hES-MSC groups

6.13 Example 12

Irradiated hES-MSCs have a Similar Lifespan to the Host Mice

[0392] The lifespan of irradiated hES-MSCs in vivo was established to determine if the irradiated cells would be effective on EAE.

Materials and Methods

[0393] To determine the lifespan of irradiated hES-MSC in vivo, CT2 hESC clone with constitutive expression of luciferase in the hESC and their progeny was produced by transducing the cells with a lentiviral vector (Pomper et al. (2009)). The cells were stained with an anti-luciferase antibody (green) and counterstained for nuclei with DAPI (blue) and were confirmed to be luciferase positive by fluorescence microscopy (FIG. 26).

[0394] The luciferase-expressing hES-MSCs (CT2) were irradiated, and non-irradiated and irradiated cells were injected into EAE mice as described in Example 2.

Results

[0395] It was found that the irradiated hES-MSC had about the same lifespan of 7-10 days in the mice post-injection as the non-irradiated hES-MSC (FIG. 27). These data, together, suggest that irradiated hES-MSC have similar lifespan in the host mice and can achieve similar efficacy on EAE (when given at doubled dose) compared to non-irradiated hES-MSC, and no tumors are found in the immune-compromised mice transplanted with hES-MSCs.

6.14 Example 14

Qualification Procedure for Clinical Grade hES-MSCs

[0396] hES-MSC are characterized through multi-color flow cytometry analyses and immunofluorescence staining using six groups of markers: (1) MSC-specific markers (set 1): CD73, CD90, CD105, CD146, CD166, and CD44, (2) MSC-specific markers (set 2): CD13, CD29, CD54, CD49E, SCA-1, and STRO-1, (3) hematopoietic stem/progenitor markers: CD45 and CD34, and endothelial cell marker CD31, (4) immunogenic markers: HLA-ABC, HLA-G, CD80, and CD86, (5) cytokines: IL-10, TGFβ, IL-6, IL-12 and TNFα, and (6) pluripotency markers: OCT4, NANOG, TRA-1-60, and SSEA-4. A clinical grade MSC contains >95% of the cells positive for group-1 markers, >80% positive for group 2, <5% for group 3, >80% positive for IL-10 and/or TGFβ, <5% positive for IL-6, IL-12 and TNFα, and <0.001% co-expressing group 6. Heterogeneity and purity of the cells can be tested as described above. The clinical-grade MSC will be compared side-by-side with the preclinical-grade MSC validated in Aim 3.1 as a positive control.

[0397] To examine whether the hES-MSC have a consistent cytokine secretion profile, 24 hr condition medium of hES-MSC will be analyzed for secreted cytokines expression using Multiplex System with R&D Fluorokine MAP multiplex Human Cytokine Panel A and TGF-beta 3-plex. All important cytokines that are critical for MSC function will be

US 2015/0203820 A1

Jul. 23, 2015

32

analyzed simultaneously with only 50-100 ul sample needed, including, but not limited to, CCL2, CCL3, CCL4, CCL5, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, TNF α , TGF β , IFN γ , IFN α , IFN β , GM-CSF, G-CSF, bFGF, CXCL5, VEGF, TPO, CXCL10, CCL11, CD40 ligand, EGF, HGF, IL12A, IL12, IL-13 and/or Leptin.

[0398] hES-MSC are also analyzed for: (1) presence of exogenous materials such as endotoxin and residual cytokines/growth factors used to differentiate hES-MSC, and (2) genomic abnormalities (via karyotyping and whole-genome sequencing).

[0399] All references, including publications, patent applications and patents, cited herein are hereby incorporated by reference to the same extent as if each reference was individually and specifically indicated to be incorporated by reference and was set forth in its entirety herein.

REFERENCES

- [0400]** Al Jumah, M. A., and Abumaree, M. H. (2012). The Immunomodulatory and Neuroprotective Effects of Mesenchymal Stem Cells (MSCs) in Experimental Autoimmune Encephalomyelitis (EAE): A Model of Multiple Sclerosis (MS). *International journal of molecular sciences* 13, 9298-9331.
- [0401]** Anton, K., Banerjee, D., and Glod, J. (2012). Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. *PLoS One* 7, e35036.
- [0402]** Auletta, J. J., Bartholomew, A. M., Maziarz, R. T., Deans, R. J., Miller, R. H., Lazarus, H. M., and Cohen, J. A. (2012). The potential of mesenchymal stromal cells as a novel cellular therapy for multiple sclerosis. *Immunotherapy* 4, 529-547.
- [0403]** Barberi, T., Willis, L. M., Socci, N. D., and Studer, L. (2005). Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PLoS Med* 2, e161.
- [0404]** Becher B, Durell B G, Noelle R J (2002) Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *The Journal of clinical investigation* 110: 493-497.
- [0405]** Benito-Leon, J. (2011). Are the prevalence and incidence of multiple sclerosis changing? *Neuroepidemiology* 36, 148-149.
- [0406]** Brown, S. E., Tong, W., and Krebsbach, P. H. (2009). The derivation of mesenchymal stem cells from human embryonic stem cells. *Cells Tissues Organs* 189, 256-260.
- [0407]** Chao, Y. X., He, B. P., and Tay, S. S. (2009). Mesenchymal stem cell transplantation attenuates blood brain barrier damage and neuroinflammation and protects dopaminergic neurons against MPTP toxicity in the substantia nigra in a model of Parkinson's disease. *Journal of neuroimmunology* 216, 39-50.
- [0408]** Chaudhary P, Marracci G H, Bourdette D N (2006) Lipoic acid inhibits expression of ICAM-1 and VCAM-1 by CNS endothelial cells and T cell migration into the spinal cord in experimental autoimmune encephalomyelitis. *Journal of neuroimmunology* 175: 87-96.
- [0409]** Chyou, S., Ekland, E. H., Carpenter, A. C., Tzeng, T. C., Tian, S., Michaud, M., Madri, J. A., and Lu, T. T. (2008). Fibroblast-type reticular stromal cells regulate the lymph node vasculature. *J Immunol* 181, 3887-3896.
- [0410]** Connick, P., Kolappan, M., Crawley, C., Webber, D. J., Patani, R., Michell, A. W., Du, M. Q., Luan, S. L., Altmann, D. R., Thompson, A. J., et al. (2012). Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of concept study. *Lancet neurology* 11, 150-156.
- [0411]** Correale, J., and Villa, A. (2007). The blood-brain-barrier in multiple sclerosis: functional roles and therapeutic targeting. *Autoimmunity* 40, 148-160.
- [0412]** Costa, M., Dottori, M., Ng, E., Hawes, S. M., Sourris, K., Jamshidi, P., Pera, M. F., Elefanti, A. G., and Stanley, E. G. (2005). The hESC line Envy expresses high levels of GFP in all differentiated progeny. *Nat Methods* 2, 259-260.
- [0413]** Cuccurullo C, Iezzi A, Fazio M L, De Cesare D, Di Francesco A, et al. (2006) Suppression of RAGE as a basis of simvastatin-dependent plaque stabilization in type 2 diabetes. *Arteriosclerosis, thrombosis, and vascular biology* 26: 2716-2723.
- [0414]** Cunnea P, McMahon J, O'Connell E, Mashayekhi K, Fitzgerald U, et al. (2010) Gene expression analysis of the microvascular compartment in multiple sclerosis using laser microdissected blood vessels. *Acta neuropathologica* 119: 601-615.
- [0415]** Dai H, Ciric B, Zhang G X, Rostami A (2012) Interleukin-10 plays a crucial role in suppression of experimental autoimmune encephalomyelitis by Bowman-Birk inhibitor. *Journal of neuroimmunology* 245: 1-7.
- [0416]** Dienz, O., and Rincon, M. (2009). The effects of IL-6 on CD4 T cell responses. *Clinical immunology* 130, 27-33.
- [0417]** Djouad, F., Ponce, P., Bony, C., Tropel, P., Appaillay, F., Sany, J., Noel, D., and Jorgensen, C. (2003). Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 102, 3837-3844.
- [0418]** Dong, C. (2008). TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat Rev Immunol* 8, 337-348.
- [0419]** Draper, J. S., Pigott, C., Thomson, J. A., and Andrews, P. W. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *Journal of anatomy* 200, 249-258.
- [0420]** Drukker, M., Katchman, H., Katz, G., Even-Tov Friedman, S., Shezen, E., Hornstein, E., Mandelboim, O., Reisner, Y., and Benvenisty, N. (2006). Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. *Stem Cells* 24, 221-229.
- [0421]** Drukker, M., Katz, G., Urbach, A., Schuldiner, M., Markel, G., Itskovitz-Eldor, J., Reubini, B., Mandelboim, O., and Benvenisty, N. (2002). Characterization of the expression of MHC proteins in human embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 99, 9864-9869.
- [0422]** English, K., Barry, F. P., Field-Corbett, C. P., and Mahon, B. P. (2007). IFN-gamma and TNF alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* 110, 91-100.
- [0423]** Ge, S., Shrestha, B., Paul, D., Keating, C., Cone, R., Guglielmotti, A., and Pachter, J. S. (2012). The CCL2 synthesis inhibitor bindarit targets cells of the neurovascular unit, and suppresses experimental autoimmune encephalomyelitis. *J Neuroinflammation* 9, 171.

US 2015/0203820 A1

Jul. 23, 2015

33

- [0424] Gijbels, K., Brocke, S., Abrams, J. S., and Steinman, L. (1995). Administration of neutralizing antibodies to interleukin-6 (IL-6) reduces experimental autoimmune encephalomyelitis and is associated with elevated levels of IL-6 bioactivity in central nervous system and circulation. *Mol Med* 1, 795-805.
- [0425] Gordon, D., Pavlovskaya, G., Glover, C. P., Uney, J. B., Wraith, D., and Scolding, N.J. (2008a). Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. *Neurosci Lett* 448, 71-73.
- [0426] Gordon, D., Pavlovskaya, G., Glover, C. P., Uney, J. B., Wraith, D., and Scolding, N.J. (2008b). Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. *Neuroscience letters* 448, 71-73.
- [0427] Gordon, D., Pavlovskaya, G., Uney, J. B., Wraith, D. C., and Scolding, N.J. (2010). Human mesenchymal stem cells infiltrate the spinal cord, reduce demyelination, and localize to white matter lesions in experimental autoimmune encephalomyelitis. *J Neuropathol Exp Neurol* 69, 1087-1095.
- [0428] Grinnemo, K. H., Mansson, A., Dellgren, G., Klingberg, D., Wardell, E., Drvota, V., Tammik, C., Holgersson, J., Ringden, O., Sylven, C., et al. (2004). Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. *J Thorac Cardiovasc Surg* 127, 1293-1300.
- [0429] Hansen, W., Westendorp, A. M., and Buer, J. (2008). Regulatory T cells as targets for immunotherapy of autoimmunity and inflammation. *Inflamm Allergy Drug Targets* 7, 217-223.
- [0430] Hofstetter, C. P., Schwarz, E. J., Hess, D., Widenfalk, J., El Manira, A., Prockop, D. J., and Olson, L. (2002). Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proceedings of the National Academy of Sciences of the United States of America* 99, 2199-2204.
- [0431] Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J., and Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* 432, 625-630.
- [0432] Hwang, N. S., Varghese, S., Lee, H. J., Zhang, Z., Ye, Z., Bae, J., Cheng, L., and Elisseeff, J. (2008). In vivo commitment and functional tissue regeneration using human embryonic stem cell derived mesenchymal cells. *Proc Natl Acad Sci USA* 105, 20641-20646.
- [0433] Huss D J, Winger R C, Cox G M, Guerau-de-Arelano M, Yang Y, et al. (2011) TGF-beta signaling via Smad4 drives IL-10 production in effector Th1 cells and reduces T-cell trafficking in EAE. *European journal of immunology* 41: 2987-2996.
- [0434] Javazon, E. H., Beggs, K. J., and Flake, A. W. (2004). Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* 32, 414-425.
- [0435] Johnston, J., and So, T. Y. (2012). First-line disease-modifying therapies in paediatric multiple sclerosis; a comprehensive overview. *Drugs* 72, 1195-1211.
- [0436] Karussis, D., Karageorgiou, C., Vaknin-Dembinsky, A., Gowda-Kurkalli, B., Gomori, J. M., Kassis, I., Bulte, J. W., Petrou, P., Ben-Hur, T., Abramsky, O., et al. (2010). Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. *Arch Neurol* 67, 1187-1194.
- [0437] Klimanskaya, I., Chung, Y., Becker, S., Lu, S. J., and Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. *Nature* 444, 481-485.
- [0438] Kurtzke J F (1983). "Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS)". *Neurology* 33 (11): 1444-52
- [0439] Leech, M. D., Barr, T. A., Turner, D. G., Brown, S., O'Connor, R. A., Gray, D., Mellanby, R. J., and Anderton, S. M. (2012). Cutting Edge: IL-6-Dependent Autoimmune Disease: Dendritic Cells as a Sufficient, but Transient, Source. *J Immunol*.
- [0440] Lin, G., Martins-Taylor, K., and Xu, R. H. (2010). Human embryonic stem cell derivation, maintenance, and differentiation to trophoblast. *Methods in molecular biology* 636, 1-24.
- [0441] Liu, R., Zhang, Z., Lu, Z., Borlongan, C., Pan, J., Chen, J., Qian, L., Liu, Z., Zhu, L., Zhang, J., et al. (2012). Human Umbilical Cord Stem Cells Ameliorate Experimental Autoimmune Encephalomyelitis by Regulating Inflammation and Remyelination. *Stem cells and development*.
- [0442] Lu, S. J., Feng, Q., Caballero, S., Chen, Y., Moore, M. A., Grant, M. B., and Lanza, R. (2007). Generation of functional hemangioblasts from human embryonic stem cells. *Nat Methods* 4, 501-509.
- [0443] Lu, S. J., Ivanova, Y., Feng, Q., Luo, C., and Lanza, R. (2009). Hemangioblasts from human embryonic stem cells generate multilayered blood vessels with functional smooth muscle cells. *Regenerative medicine* 4, 37-47.
- [0444] Lu, S. J., Luo, C., Holton, K., Feng, Q., Ivanova, Y., and Lanza, R. (2008). Robust generation of hemangioblastic progenitors from human embryonic stem cells. *Regen Med* 3, 693-704.
- [0445] Ludwig T E, Levenstein M E, Jones J M, Berggren W T, Mitchen E R, et al. (2006) Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* 24: 185-187.
- [0446] Mahad D J, Ransohoff R M (2003) The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). *Seminars in immunology* 15: 23-32.
- [0447] McFarland, H. F., and Martin, R. (2007). Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 8, 913-919.
- [0448] Menge, T., Zhao, Y., Zhao, J., Wataha, K., Gerber, M., Zhang, J., Letourneau, P., Redell, J., Shen, L., Wang, J., et al. (2012). Mesenchymal Stem Cells Regulate Blood-Brain Barrier Integrity Through TIMP3 Release After Traumatic Brain Injury. *Science translational medicine* 4, 161ra150.
- [0449] Minagar, A., Maghzi, A. H., McGee, J. C., and Alexander, J. S. (2012). Emerging roles of endothelial cells in multiple sclerosis pathophysiology and therapy. *Neurological research* 34, 738-745.
- [0450] Mohyeddin Bonab, M., Yazdanbakhsh, S., Lotfi, J., Alimoghaddom, K., Talebian, F., Hooshmand, F., Ghamvazadeh, A., and Nikbin, B. (2007). Does mesenchymal stem cell therapy help multiple sclerosis patients? Report of a pilot study. *Iranian journal of immunology: IJI* 4, 50-57.
- [0451] Morando, S., Vigo, T., Esposito, M., Casazza, S., Novi, G., Principato, M. C., Furlan, R., and Uccelli, A. (2012). The therapeutic effect of mesenchymal stem cell transplantation in experimental autoimmune encephalo-

US 2015/0203820 A1

Jul. 23, 2015

34

- myelitis is mediated by peripheral and central mechanisms. *Stem Cell Res Ther* 3, 3.
- [0452] Ohtaki, H., Ylostalo, J. H., Foraker, J. E., Robinson, A. P., Reger, R. L., Shioda, S., and Prockop, D. J. (2008). Stem/progenitor cells from bone marrow decrease neuronal death in global ischemia by modulation of inflammatory/immune responses. *Proc Natl Acad Sci USA* 105, 14638-14643.
- [0453] Olivier, E. N., Rybicki, A. C., and Bouhassira, E. E. (2006). Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. *Stem Cells* 24, 1914-1922.
- [0454] Patanella, A. K., Zinno, M., Quaranta, D., Nociti, V., Frisullo, G., Gainotti, G., Tonali, P. A., Batocchi, A. P., and Marra, C. (2010). Correlations between peripheral blood mononuclear cell production of BDNF, TNF-alpha, IL-6, IL-10 and cognitive performances in multiple sclerosis patients. *J Neurosci Res* 88, 1106-1112.
- [0455] Payne, N. L., Sun, G., McDonald, C., Layton, D., Moussa, L., Emerson-Webber, A., Veron, N., Siatskas, C., Herszfeld, D., Price, J., et al. (2012). Distinct immunomodulatory and migratory mechanisms underpin the therapeutic potential of human mesenchymal stem cells in autoimmune demyelination. *Cell Transplant*.
- [0456] Peron, J. P., Jazedje, T., Brandao, W. N., Perin, P. M., Maluf, M., Evangelista, L. P., Halpern, S., Nisenbaum, M. G., Czeresnia, C. E., Zatz, M., et al. (2012). Human endometrial-derived mesenchymal stem cells suppress inflammation in the central nervous system of EAE mice. *Stem Cell Rev* 8, 940-952.
- [0457] Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosea, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143-147.
- [0458] Pomper, M. G., Hammond, H., Yu, X., Ye, Z., Foss, C. A., Lin, D. D., Fox, J. J., and Cheng, L. (2009). Serial imaging of human embryonic stem-cell engraftment and teratoma formation in live mouse models. *Cell Res* 19, 370-379.
- [0459] Quintana, A., Muller, M., Frausto, R. F., Ramos, R., Getts, D. R., Sanz, E., Hofer, M. J., Krauthausen, M., King, N. J., Hidalgo, J., et al. (2009). Site-specific production of IL-6 in the central nervous system retargets and enhances the inflammatory response in experimental autoimmune encephalomyelitis. *Journal of immunology* 183, 2079-2088.
- [0460] Rafei, M., Birman, E., Forner, K., and Galipeau, J. (2009a). Allogeneic mesenchymal stem cells for treatment of experimental autoimmune encephalomyelitis. *Mol Ther* 17, 1799-1803.
- [0461] Rafei, M., Campeau, P. M., Aguilar-Mahecha, A., Buchanan, M., Williams, P., Birman, E., Yuan, S., Young, Y. K., Boivin, M. N., Forner, K., et al. (2009b). Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. *J Immunol* 182, 5994-6002.
- [0462] Rochman, I., Paul, W. E., and Ben-Sasson, S. Z. (2005). IL-6 increases primed cell expansion and survival. *Journal of immunology* 174, 4761-4767.
- [0463] Ryan, J. M., Barry, F., Murphy, J. M., and Mahon, B. P. (2007). Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp Immunol* 149, 353-363.
- [0464] Sanchez, L., Gutierrez-Aranda, I., Ligerio, G., Rubio, R., Munoz-Lopez, M., Garcia-Perez, J. L., Ramos, V., Real, P. J., Bueno, C., Rodriguez, R., et al. (2011). Enrichment of human ESC derived multipotent mesenchymal stem cells with immunosuppressive and anti-inflammatory properties capable to protect against experimental inflammatory bowel disease. *Stem Cells* 29, 251-262.
- [0465] Sethe, S., Scutt, A., and Stolzing, A. (2006). Aging of mesenchymal stem cells. *Ageing Res Rev* 5, 91-116.
- [0466] Solchaga, L. A., Penick, K. J., and Welter, J. F. (2011). Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks. *Methods in molecular biology* 698, 253-278.
- [0467] Stromnes, I. M., and Gorman, J. M. (2006). Active induction of experimental allergic encephalomyelitis. *Nat Protoc* 1, 1810-1819.
- [0468] Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-1147.
- [0469] Tse, W. T., Pendleton, J. D., Beyer, W. M., Egalka, M. C., and Guinan, E. C. (2003). Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 75, 389-397.
- [0470] Tyndall, A. (2011). Successes and failures of stem cell transplantation in autoimmune diseases. *Hematology Am Soc Hematol Educ Program* 2011, 280-284.
- [0471] Uccelli, A., and Prockop, D. J. (2010a). Why should mesenchymal stem cells (MSCs) cure autoimmune diseases? *Curr Opin Immunol* 22, 768-774.
- [0472] Uccelli, A., and Prockop, D. J. (2010b). Why should mesenchymal stem cells (MSCs) cure autoimmune diseases? *Curr Opin Immunol*, 7.
- [0473] Waterman, R. S., Tomchuck, S. L., Henkle, S. L., and Betancourt, A. M. (2010). A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS One* 5, e10088.
- [0474] Weber, M. S., Menge, T., Lehmann-Horn, K., Kronsbein, H. C., Zettl, U., Selinger, J., Hemmer, B., and Stave, O. (2012). Current treatment strategies for multiple sclerosis—efficacy versus neurological adverse effects. *Current pharmaceutical design* 18, 209-219.
- [0475] Wong, R. S. (2011). Mesenchymal stem cells: angels or demons? *J Biomed Biotechnol* 2011, 459510.
- [0476] Yamout, B., Hourani, R., Salti, H., Barada, W., El-Hajj, T., Al-Kutoubi, A., Herlopian, A., Baz, E. K., Mahfouz, R., Khalil-Hamdan, R., et al. (2010). Bone marrow mesenchymal stem cell transplantation in patients with multiple sclerosis: a pilot study. *J Neuroimmunol* 227, 185-189.
- [0477] Zappia, E., Casazza, S., Pedemonte, E., Benvenuto, F., Bonanni, I., Gerdoni, E., Giunti, D., Ceravolo, A., Cazzanti, F., Frasson, F., et al. (2005). Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106, 1755-1761.
- [0478] Zhang, J., Li, Y., Chen, J., Cui, Y., Lu, M., Elias, S. B., Mitchell, J. B., Hammill, L., Vanguri, P., and Chopp, M. (2005). Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Exp Neurol* 195, 16-26.

US 2015/0203820 A1

Jul. 23, 2015

35

- [0479] Becher, B., Durell, B. G., and Noelle, R. J. (2002). Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *The Journal of clinical investigation* 110, 493-497.
- [0480] Chaudhary, P., Marracci, G. H., and Bourdette, D. N. (2006). Lipoic acid inhibits expression of ICAM-1 and VCAM-1 by CNS endothelial cells and T cell migration into the spinal cord in experimental autoimmune encephalomyelitis. *Journal of neuroimmunology* 175, 87-96.
- [0481] Crocker, S. J., Milner, R., Pham-Mitchell, N., and Campbell, I. L. (2006). Cell and agonist-specific regulation of genes for matrix metalloproteinases and their tissue inhibitors by primary glial cells. *Journal of neurochemistry* 98, 812-823.
- [0482] Cuccurullo, C., Iezzi, A., Fazio, M. L., De Cesare, D., Di Francesco, A., Muraro, R., Bei, R., Uchino, S., Spigonardo, F., Chiarelli, F., et al. (2006). Suppression of RAGE as a basis of simvastatin-dependent plaque stabilization in type 2 diabetes. *Arteriosclerosis, thrombosis, and vascular biology* 26, 2716-2723.
- [0483] Cunnea, P., McMahon, J., O'Connell, E., Mashayekhi, K., Fitzgerald, U., and McQuaid, S. (2010). Gene expression analysis of the microvascular compartment in multiple sclerosis using laser microdissected blood vessels. *Acta neuropathologica* 119, 601-615.
- [0484] Dai, H., Ciric, B., Zhang, G. X., and Rostami, A. (2012). Interleukin-10 plays a crucial role in suppression of experimental autoimmune encephalomyelitis by Bowman-Birk inhibitor. *Journal of neuroimmunology* 245, 1-7.
- [0485] Huss, D. J., Winger, R. C., Cox, G. M., Guerau-de-Arellano, M., Yang, Y., Racke, M. K., and Lovett-Racke, A. E. (2011). TGF-beta signaling via Smad4 drives IL-10 production in effector Th1 cells and reduces T-cell trafficking in EAE. *Eur J Immunol* 41, 2987-2996.
- [0486] Mahad, D. J., and Ransohoff, R. M. (2003). The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). *Semin Immunol* 15, 23-32.
- [0487] Moore, C. S., Milner, R., Nishiyama, A., Frausto, R. F., Serwanski, D. R., Pagarigan, R. R., Whitton, J. L., Miller, R. H., and Crocker, S. J. (2011). Astrocytic tissue inhibitor of metalloproteinase-1 (TIMP-1) promotes oligodendrocyte differentiation and enhances CNS myelination. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 31, 6247-6254.
- [0488] Quintana, A., Muller, M., Frausto, R. F., Ramos, R., Getts, D. R., Sanz, E., Hofer, M. J., Krauthausen, M., King, N. J., Hidalgo, J., et al. (2009). Site-specific production of IL-6 in the central nervous system retargets and enhances the inflammatory response in experimental autoimmune encephalomyelitis. *Journal of immunology* 183, 2079-2088.
1. A method for producing human embryonic stem cell-derived mesenchymal stem cells, comprising:
 - a. culturing human embryonic stem cells with serum free media with or without GSK3 inhibitors;
 - b. culturing human embryonic stem cells in serum-free media comprising vascular endothelial growth and bone morphogenic protein 4 (BMP4) in an amount sufficient to induce differentiation into embryoid bodies;
 - c. adding at least one growth factor to the culture, wherein the growth factor is in an amount sufficient to expand human hemangio-colony forming cells;
 - d. disaggregating the hemangio-colony forming cells into single cells; and
 - e. culturing the single cells in media containing serum, KOSR or other serum-free medium in an amount sufficient to induce differentiation of the single cells into mesenchymal stem cells;
 wherein at least about 90% of the mesenchymal stem cells express CD73, said MSC having the following characteristics: (i) contain >95% of cells expressing group-1 markers; (ii) contain >80% of cells expressing group 2 markers; (iii) contain <5% of cells expressing group-3 markers (iv) expressing IL-10 and TGF β ; (v) contain <2% of cells expressing IL-6, IL-12 and TNF α ; and (vi) contains <0.001% of cells co-expressing all group-4 markers, wherein group-1 markers are CD73, CD90, CD105, CD146, CD166, and CD44, group-2 markers are CD13, CD29, CD54, CD49E, group-3 markers are CD45, CD34, CD31 and SSEA4, and group-4 markers are OCT4, NANOG, TRA-1-60 and SSEA4.
 2. The method of claim 1, wherein the cells do not express IL-6, IL12 and TNF α .
 3. The method of claim 1, wherein the cells express TGF-beta1, TGF-beta2 and IL10.
 4. The method of claim 1, wherein the cells do not express CCL2, MMP2 and RAGE
 5. The method of claim 1, where in the cells have low expression of IFN γ R1, IFN γ R2 as compared to BM-MSC.
 6. A method of modifying mesenchymal stem cells to produce a population of modified MSC having the following characteristics: (i) contain >95% of cells expressing group-1 markers; (ii) contain >80% of cells expressing group 2 markers; (iii) contain <5% of cells expressing group-3 markers (iv) expressing IL-10 and TGF β ; (v) contain <2% of cells expressing IL-6, IL-12 and TNF α ; and (vi) contains <0.001% of cells co-expressing all group-4 markers, wherein group-1 markers are CD73, CD90, CD105, CD146, CD166, and CD44, group-2 markers are CD13, CD29, CD54, CD49E, group-3 markers are CD45, CD34, CD31 and SSEA4, and group-4 markers are OCT4, NANOG, TRA-1-60 and SSEA4.
 - 7-12. (canceled)
 13. The method of claim 1, further comprising a step of irradiating the mesenchymal stem cells.
 - 14-18. (canceled)
 19. A cell culture comprising the hES-MSC prepared by the method of claim 1.
 - 20-22. (canceled)
 23. A pharmaceutical preparation comprising the hES-MSC prepared by the method of claim 1 and a pharmaceutical acceptable carrier.
 - 24-26. (canceled)
 27. A method for preventing or treating a T cell and/or B cell related autoimmune disease comprising administering to a subject in need thereof the hES-MSC prepared by the method of claim 1, an amount sufficient to ameliorate or alleviate at least one symptom of the T cell related autoimmune disease or reverse the T cell and/or B cell related autoimmune disease.
 - 28-42. (canceled)
 43. A method for preventing or treating a multiple sclerosis comprising administering to a subject in need thereof the hES-MSC prepared by the method of claim 1, an amount sufficient to ameliorate or alleviate at least one symptom of the disease or reverse the disease.

US 2015/0203820 A1

Jul. 23, 2015

36

44-58. (canceled)

59. A method of delivering an agent through the blood-brain barrier and/or the blood-spinal cord barrier, said method comprising the steps of: attaching the agent to a MSC to form a MSC-agent complex; and administering the MSC-agent complex to a subject in need thereof, wherein the human embryonic-mesenchymal stem cell is capable of crossing the blood-brain barrier and/or the blood-spinal cord barrier and the agent is for the treatment, prevention or diagnosis of a disease or injury in the subject in need thereof.

60. A kit comprising the hES-MSC prepared by the method of claim **1** and a carrier.

61. The kit of claim **60** further comprising a thawing reagent, immunosuppressive enhancer, anti-histamine.

62. The method of claim **13** wherein the mesenchymal stem cells are irradiated with gamma-irradiation.

63-66. (canceled)

67. The method of claim **1** wherein the hES-MSC are modified by genetic modification, epigenetic regulation, small molecule, nutraceutical, natural compound, or antibody treatment.

68-82. (canceled)

83. The method of claim **1** further comprising co-culturing the hES-MSC with bone marrow hematopoietic stem cell and/or umbilical-cord hematopoietic stem cell.

84-87. (canceled)

88. The method of claim **83** wherein the hES-MSC is co-cultured with hematopoietic stem cells.

89-92. (canceled)

* * * * *